ROLE OF PROTEINASE-ACTIVATED RECEPTOR-1-DEPENDENT SECONDARY MEDIATORS IN LUNG FIBROSIS

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A thesis submitted for the degree of PhD at the Universiy of London,

October 2007

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Acknowledgements

Thank you to: my PhD supervisor, Dr Rachel Chambers for expert training, supervision, guidance and support; Professor Geoffrey Laurent, who invited me to join the Centre for Respiratory Research, in which this work was undertaken; Mr Oliver Prenn, who funded my first eight months in the centre, and the MRC who then funded the remainder of my studies with a Clinical Research Training Fellowship; All the collaborators who helped with this work, in particular, Professors Terry Tetley, and Joseph Lasky; Mr Steve Bottoms for extensive teaching and advice on histological techniques; Dr Chris Scotton, Dr Paul Mercer, Dr David Howell, Dr Gary Lee and Dr Pat Garcia for invaluable scientific advice and support; and all the other colleagues I had the pleasure to work with and learn from during the course of this PhD.

Declaration

I confirm that the work contained in this thesis is entirely my own.

ABSTRACT

Persistent local activation of the coagulation cascade typifies fibroproliferative lung disorders such as idiopathic pulmonary fibrosis and the acute respiratory distress syndrome. In addition to its role in coagulation, thrombin exerts potent pro-inflammatory and pro-fibrotic effects via activation of its major cellular receptor proteinase-activated receptor-1 (PAR_1). In this thesis, the relevance of PAR_1 activation to inflammation and fibrosis in a model of lung injury and fibrosis based on the intra-tracheal instillation of bleomycin was examined. Lung inflammation and fibrosis were substantially attenuated in PAR_1-deficient mice. The attenuated inflammatory response was manifest by substantial reductions in bronchoalveolar lavage protein content, total leukocyte numbers, and lung levels of the chemokine CCL2. The attenuated fibrotic response was manifest by a reduction in lung collagen accumulation of around 60%, and reduced mRNA and/or protein levels of the pro-fibrotic mediators connective tissue growth factor (CTGF) and transforming growth factor-beta-1 (TGF-β_1). These data suggest that PAR_1 may promote inflammation and fibrosis, at least in part, via the expression of CCL2, CTGF, and TGFβ_1. Further studies demonstrated prominent immunostaining for PAR_1 on lung epithelial cells following bleomycin injury, consistent with these cells being a potential source of PAR_1-dependent secondary mediators in the injured lung. Widespread epithelial injury is a prominent feature of fibroproliferative lung disorders, in which hyperplastic activated epithelial cells represent a major source of pro-inflammatory and pro-fibrotic mediators. The effect of PAR_1 activation on lung epithelial expression of CCL2, CTGF and TGFβ_1 was therefore examined *in vitro*. Exposure to both thrombin and the PAR_1 agonist peptide TFLLR-NH_2 substantially increased mRNA levels of CCL2 and CTGF, but not TGFβ_1 in A549 and BEAS-2B human lung epithelial cells. Increases in CCL2 and CTGF mRNA levels in response to thrombin were attenuated with the PAR_1 antagonist RWJ-58259. PAR_1-dependent increases in CCL2 and CTGF mRNA are apparently protein kinase C-dependent since increases in response to TFLLR-NH_2 were inhibited by the protein kinase C inhibitor Ro-31-8425. Increases in CCL2 and CTGF mRNA in response to PAR_1 activation were mirrorred by comparable increases at the protein level. Taken together, these data suggest that epithelial cells may represent a source of PAR_1-dependent pro-inflammatory and pro-fibrotic mediators following lung injury.

Table of Contents

List of Tables

List of Figures

1. INTRODUCTION

1.1. Pulmonary fibrosis

Pulmonary fibrosis is a pathological process characterized by excessive and disorganised deposition of extracellular matrix proteins, such as collagen within the pulmonary interstitium. This has a profound detrimental impact on gas exchange, and is responsible for considerable morbidity and mortality in patients. Pulmonary fibrosis is common to a number of interstitial lung disorders which may have distinct aetiologies and pathophysiology. Such conditions include those resulting from exposure to radiation, dust, infectious agents, allergens, or chemicals. In addition, pulmonary fibrosis may be a feature of diseases which progressively affect the lungs as one part of systemic involvement. Examples of this are systemic sclerosis, rheumatoid arthritis and sarcoidosis. Conversely, pulmonary fibrosis may occur rapidly following acute severe insults to the lung. Examples of this are the acute respiratory distress syndrome (ARDS) which can occur in response to septicaemia or various other insults, and bronchopulmonary dysplasia which can occur in premature infants. This latter probably results in part from the effect of high oxygen concentrations and shear stresses associated with mechanical ventilation on underdeveloped lungs. Pulmonary fibrosis is also a hallmark feature of the idiopathic interstitial pneumonias. There are seven separate clinicopathological categories of interstitial pneumonia. Diagnosis of these is based on clinical, radiological, and histological findings. The categories of interstitial pneumonia are as shown in **table 1.1.**

Histological pattern	**Diagnosis based on clinical, radiological, and histological findings**
Usual interstitial pneumonia (UIP)	Idiopathic pulmonary fibrosis (IPF)
Nonspecific interstitial pneumonia (NSIP)	Nonspecific interstitial pneumonia (NSIP)
Organizing pneumonia	Cryptogenic organizing pneumonia (COP)
Diffuse alveolar damage	Acute interstitial pneumonia (AIP)
Respiratory bronchiolitis	Respiratory bronchiolitis-associated interstitial lung disease (RBILD)
Desquamative interstitial pneumonia	Desquamative interstitial pneumonia (DIP)
Lymphoid interstitial pneumonia	Lymphoid interstitial pneumonia (LIP)

Table 1.1. Classification of the idiopathic interstitial pneumonias (Table adapted from (Khalil & O'Connor 2004))

1.1.1. Idiopathic pulmonary fibrosis (IPF)

IPF is the commonest of the idiopathic interstitial pneumonias. The peak incidence occurs in the sixth decade of life and the disease is more common in males than females. Reported estimates of incidence and prevalence in the literature vary; but incidence may be 7-10 cases per 100,000/year, and prevalence may be 20 per 100,000 (Coultas et al. 1994). Several risk factors such as cigarette smoking (Baumgartner et al. 1997) and viral infection (Tang et al. 2003) have been proposed. The existence of familial cases of idiopathic interstitial pneumonia suggests there may be a genetic basis, and various polymorphisms, such as of surfactant protein C, associate with an increased incidence of IPF (Lawson et al. 2004). Nevertheless, most individuals who *do* develop IPF currently have no known predisposing risk factor.

The pathophysiological basis of IPF has been the subject of much debate over the last few decades. Previously it was widely thought that chronic inflammation injured the lung, which subsequently initiated widespread fibrogenesis. However, given the limited evidence for persistent inflammation in progressive lung fibrosis,

and the lack of efficacy of anti-inflammatory therapeutic interventions, there is growing consensus that fibrogenesis results, at least in part, from multi-focal epithelial microinjury with subsequent disordered epithelial repair and abnormal epithelial-fibroblastic interactions (Reviewed in (Selman et al. 2001)). The contribution of epithelial cells to fibrogenesis will be discussed in more depth in a following section: **1.5.2. Epithelial injury within the lung**.

Clinically, the symptoms of IPF are dyspnoea and an unproductive cough. Physical signs on examination are usually inspiratory crackles at the lung bases, sometimes with digital clubbing. Lung biopsy typically demonstrates a histological pattern known as Usual Interstitial Pneumonia (UIP), which is one of interstitial fibrosis containing areas of dense scarring, honeycombing, and scattered fibroblastic foci, often alternating with areas of normal lung. Radiological imaging demonstrates peripheral subpleural lower zone reticular opacities, possibly together with subpleural honeycombing, traction bronchiectasis and limited ground-glass opacity. Lung function tests demonstrate a restrictive airflow defect, reduced lung volumes, reduced carbon monoxide gas transfer and arterial hypoxaemia.

In many patients the clinical course after diagnosis is one of relentlessly progressive respiratory impairment and dyspnoea leading ultimately to respiratory failure. In a study of newly diagnosed cases of IPF (Hubbard et al. 1998), the median survival was only 2.9 years, although it is now apparent that a number of clinical, radiological, and physiological parameters have useful prognostic value in predicting survival in individual patients (King, Jr. et al. 2001).

Current therapeutic options for IPF essentially comprise corticosteroids, and immunosuppressive/cytotoxic agents (e.g. azathioprine, cyclophosphamide). Unfortunately, these treatments have made little impact on morbidity and mortality. However, improvements in our understanding of the biochemical and biological mechanisms of lung fibrosis have led to the development of a number of novel therapies, which are currently being evaluated. These will be discussed in a later section: **1.1.5. Novel therapies for IPF**.

1.1.2. The acute respiratory distress syndrome

The acute respiratory distress syndrome (ARDS) is a syndrome comprising noncardiogenic pulmonary oedema and respiratory failure, and occurring within hours

to days of a predisposing insult. It may occur in the context of *direct* lung injury, such as from pneumonia or aspiration, as well as in the context of *indirect* lung injury associated with trauma, sepsis, and other conditions including pancreatitis, drug overdose, or multiple blood transfusions. There is also some evidence to suggest that certain polymorphisms in inflammatory genes such as tumour necrosis factor-alpha (TNF-α) (Gong et al. 2005) and interleukin-6 (IL-6) (Marshall et al. 2002b), angiotensin converting enzyme (ACE) (Marshall et al. 2002a), and surfactant proteins (Lin et al. 2000) may influence the incidence and outcome of ARDS and/or sepsis.

In a Scandinavian multi-centre prospective study (Luhr et al. 1999), the incidence of ARDS in the general population was found to be 13.5/100000/year. 221 (14.6%) of 1515 of ICU patients that required either at least twenty four hours of care, or oxygen therapy (with a fraction of inspired oxygen (FiO_2) of at least 0.4) and ventilatory support, had ARDS. The mean age of these ARDS patients was 61.3 ± 16.5 years, and the mortality in the first 90 days following diagnosis was 41.2%.

The pathogenesis of ARDS involves initial widespread injury to epithelial and endothelial surfaces leading to disruption of the alveolar-capillary unit. The ensuing "diffuse alveolar damage" is considered to comprise exudative, proliferative, and fibrotic phases. The exudative phase is characterised by leakage of protein-rich fluid from capillaries into the alveolar space. This promotes the formation of hyaline membranes, containing condensed plasma proteins and fibrin, within the alveoli. Alveolar septa are also engorged as a result of interstitial oedema, and extravasated erythrocytes, inflammatory cells, and fibrin. The proliferative phase comprises proliferation of type II alveolar epithelial cells and fibroblasts, whilst the fibrotic phase is characterised by the synthesis of matrix proteins such as collagens I and III. This results in alveolar fibrosis, but may also lead to vascular wall remodelling and pulmonary arterial hypertension. In fact these phases may overlap temporally, rather than follow sequentially, and there is evidence that collagen synthesis may occur within the first 24 hours of mechanical ventilation in patients with acute lung injury (reviewed in (Marshall et al. 1998)).

Clinically, ARDS is manifest by impaired arterial oxygenation, and bilateral pulmonary infiltrates on chest X-ray, in the absence of left ventricular heart failure. Management is largely supportive and includes oxygen therapy combined with ventilatory, haemodynamic, and nutritional support. Vasodilators such as nitric oxide or prostacyclin may be useful in those patients with co-exisiting pulmonary arterial

hypertension. Instillation of surfactant has been shown to improve survival in premature infants, but not in adults with acute respiratory distress syndrome. Most deaths from ARDS occur in the first two weeks after diagnosis. Recovery in survivors can take several months. Interestingly much of the collagen deposited within the lung seems to disappear in these individuals. Nonetheless, many of these patients are left with residual impairment in respiratory function, and reduced ability to carry out normal activities of daily living (Heyland et al. 2005).

1.1.3. Mechanisms of pulmonary fibrosis

Multiple mediators and complex mechanisms promote the development of fibrosis in fibroproliferative lung disorders such as IPF and ARDS. However a central early event in both IPF and ARDS is widespread epithelial cell injury and necrosis of type I alveolar epithelial cells. The role of epithelial injury in fibrogenesis will be considered in a later section: **1.5.2. Epithelial injury within the lung**. The resulting compromise of alveolar capillary wall integrity permits the formation of a provisional fibrinous matrix on denuded basement membrane of the alveolar wall, reactive hyperplasia and proliferation of type II alveolar and possibly bronchiolar epithelial cells, and their production of a number of mediators which drive proliferation, activation and migration of underlying fibroblasts into the provisional matrix. These events effectively represent an excessive and aberrant healing response. The outcome is dependent on the nature and extent of epithelial injury, but is also affected by other influences such as the local oxidant/ antioxidant balance. Antioxidants such as glutathione protect cells from injurious reactive oxygen species and their levels are reduced in the epithelial lining fluid in IPF (Cantin et al. 1989).

The formation of a provisional fibrinous matrix on the damaged alveolar wall is enabled by the local upregulation of tissue factor (TF), the initiator of coagulation. This promotes the generation of thrombin which then forms insoluble fibrin from plasma fibrinogen. Under the influence of pro-fibrotic mediators, neighbouring fibroblasts migrate into the provisional matrix and the adjacent interstitium, proliferate, and transdifferentiate into contractile, activated myofibroblasts which synthesize and deposit abundant extracellular matrix proteins, including collagens types I and III. Localised groups of highly active collagen-synthesizing myofibroblasts form fibroblastic foci, which are the histological hallmarks of UIP.

Examples of mediators implicated in lung fibrosis are shown in **table 1.2.** The most potent of these mediators is undoubtedly transforming growth factor-beta-1 (TGF-β_1); and this together with the chemokine CCL2 and connective tissue growth factor (CTGF) will be considered in more depth in later sections. Notably the pro-fibrotic effects of a number of these mediators may be counteracted by various protective or anti-fibrotic mediators including interferon gamma (IFN-γ), prostaglandin E_2 (PGE_2), and the antioxidant glutathione. In general, fibrosis of the lung and other organs involves a shift in the local balance of Th_1/Th_2-type cytokines in favour of a Th_2 profile. Th_2 cytokines such as IL-4 and IL-13 promote fibrosis by stimulating fibroblast collagen synthesis (Gillery et al. 1992),(Oriente et al. 2000) whilst Th_1 cytokines such as IFN-γ may inhibit fibroblast function and collagen synthesis (Narayanan et al. 1992) and are thus anti-fibrotic.

Chemokines
CXCL5 (Epithelial neutrophil activating protein-78 (ENA-78))
CXCL8 (interleukin-8)
CCL2 (Monocyte chemoattractant protein-1 (MCP-1))
CCL3 (Macrophage inflammatory protein-1-alpha (MIP-1α)
Leukotrienes
LTB4, LTC4 (Leukotrienes C4, E4)
Cytokines
IL-1b
IL-4
IL-13
TNF-α
ET-1 (Endothelin-1)
Growth factors
TGF-β_1
TGF-α
CTGF
PDGF (Platelet-derived growth factor)
IGF-1 (Insulin-like growth factor-1)
Coagulation proteinases
Thrombin Factor Xa (FXa)
Protease inhibitors
PAI-1 (Plasminogen activator inhibitor-1)
TIMP-1 (Tissue inhibitor of metalloproteinase-1)

Table 1.2. Mediators implicated in lung fibrosis

Persistence of extracellular matrix components is dependent on the balance of factors which promote their synthesis and those which promote their degradation. Fibrin persistence may result from increased levels of plasminogen activator inhibitor-1 (PAI-1) in IPF, leading to inhibition of plasmin activation, and reduced fibrin degradation (Fujii et al. 2000). Matrix metalloproteinases (MMPs) are important in the degradation of matrix proteins such as collagen. In IPF levels of endogenous MMP inhibitors: tissue inhibitors of matrix metalloproteinases (TIMPs) appear to be upregulated to a greater extent than MMPs such that matrix components persist (Selman et al. 2000).

In a normal healing response, following deposition of limited extracellular matrix in the wound space, myofibroblasts contract to close the wound and then disappear by apoptosis. Epithelial cell proliferation then ensues to re-establish a covering epithelial lining. In lung fibrosis the opposite situation occurs such that fibroblast/myofibroblast apoptosis is reduced but epithelial cell apoptosis and necrosis is increased. Reduced fibroblast apoptosis may occur in response to increased levels of TGF-β_1 (Jelaska & Korn 2000), whereas increased epithelial apoptosis may occur in response to fibroblast-derived angiotensin peptides (Uhal et al. 1995).

There has been some controversy as to the importance of inflammatory cells in fibrogenesis in pulmonary fibrosis. Cellular inflammation is a prominent histological feature in non-specific interstitial pneumonia (NSIP) but not UIP. However the presence of both pathologies in biopsies of the same lobe of lung in number of patients with idiopathic interstitial pneumonia suggests that NSIP may evolve temporally into UIP, and that the two entities are in fact part of the same disease process (Strieter 2002). Thus inflammation may play a role in initiating epithelial injury and promoting fibroblast function in early disease by the production of pro-inflammatory and pro-fibrotic growth factors. In bleomycin-induced lung fibrosis in rodents, inflammatory cells represent a potential source of important pro-inflammatory mediators such as IL-1b (Maeda et al. 1996) and TNF-α (Everson & Chandler 1992), and pro-fibrotic mediators such as CCL2 (Sakanashi et al. 1994), IGF-1, PDGF (Maeda et al. 1996), and TGF-β_1 (Khalil et al. 2002). Furthermore IL-1b, TNF-α, and the Th_2 cytokine IL-13 can provide a link between inflammation and fibrosis since these pro-inflammatory agents are able, either individually or in combination, to induce expression of pro-fibrotic TGF-β_1 *in vitro* (Yue et al.

1994),(Warshamana et al. 2001),(Fichtner-Feigl et al. 2006). In addition, overexpression of these mediators in mice promotes increased lung TGF-β_1 levels and fibrosis (Kolb et al. 2001),(Sime et al. 1998),(Lee et al. 2001).

In UIP, however, the paucity of inflammatory cells and the lack of efficacy of anti-inflammatory therapies argue against inflammation being important, at least in advanced disease. In this setting, the major cellular source of pro-inflammatory and pro-fibrotic mediators may comprise hyperplastic epithelial cells (Kapanci et al. 1995). Conversely, inflammatory cells *are* a more prominent feature in ARDS than in advanced IPF, and are more likely to contribute to fibrogenesis in ARDS, although hyperplastic epithelial cells remain an additional key source source of pro-inflammatory and pro-fibrotic mediators.

Finally two further mechanisms may contribute to fibrosis, namely fibrocyte recruitment and epithelial-mesenchymal transition. Fibrocytes are circulating cells derived from bone marrow that express both leukocyte (CD45, CD13) and mesenchymal (Col 1) markers. They exhibit chemotaxis to chemokines such as CCL2 (Moore et al. 2005) and CCL12 (Moore et al. 2006), and evidence from animal models suggests they are recruited to the injured lung and promote fibrosis by synthesizing TGF-β_1 and extracellular matrix components such as collagen. Finally, collagen-synthesizing mesenchymal cells may also be derived from type II alveolar epithelial cells. In a process known as epithelial-mesenchymal transition, alveolar epithelial cells chronically exposed to the profibrotic mediator TGF-β_1 transdifferentiate to a myofibroblast-like phenotype. This is associated with *de novo* expression of the mesenchymal marker Fibronectin-EDA (Fn-EDA) and a concomitant downregulation of the epithelial marker E-cadherin (E-cad) (Willis et al. 2005),(Kasai et al. 2005).

1.1.4. Collagen synthesis in pulmonary fibrosis

Resident cells within all organs synthesize a range of extracellular matrix proteins. These proteins provide structural integrity for tissues and serve to protect cells from damaging mechanical influences. They also exert important influences over cellular function. For example they enable cell attachment and migration, influence cell differentiation and gene expression, allow mechanically transduced signal

transduction, and serve as important reservoirs of growth factors by virtue of these being able to bind certain matrix components. Extracellular matrix is thus crucial to the normal functioning of organs, and abnormal extracellular matrix composition or disordered architecture has profound deleterious consequences.

Collagens are the most abundant proteins present in extracellular matrix in both normal and fibrotic tissues (reviewed in (Gelse et al. 2003)). There are twenty eight known types of collagen. Each comprises a characteristic triple helix consisting of 3 polypeptide chains. Type IV collagen serves as an important structural component of basement membranes and comprises heterotimers of six possible subunit chains. Type I and III collagens assemble into large orientated supramolecular aggregates known as fibrils. Type I collagen is the major protein constituent of bone, tendon, ligaments and skin. Type III collagen is an important constituent of reticular fibres within the interstitium of the lungs, liver, dermis and spleen. The presence of types I and III collagen within the lung is crucial in providing structural integrity in an organ which contains intricate and extensive networks of airspaces and capillaries, and is exposed to considerable mechanical stresses.

Type I collagen is the most abundant of the collagens and is usually a heterotrimer of 2 α_1(I) chains and 1 α_2(I) chain. An important mechanism by which factors such as TGF-β_1, TNF-α, IL-4, IL-13, and thrombin promote fibrosis is by induction of procollagen α_1(I) gene expression (Hetzel et al. 2005),(Gillery et al. 1992),(Oriente et al. 2000),(Chambers et al. 1998). Type III collagen is a homotrimer of 3 α_1(III) chains, and is often incorporated into composite fibrils with type I collagen.

Collagen genes involve a complex intron-exon pattern. Hence mRNA species of differing lengths can result from alternative splicing and use of different transcription initiation sites. Translation leads to the synthesis of preprocollagen which is converted to procollagen by removal of its signal peptide. Procollagen molecules then undergo extensive post-translational modification and incorporation into triple helices within the Golgi compartment. They are released from here via secretory vesicles into the extracellular space, where N and C-terminal peptides are cleaved off and assembly into fibrils occurs.

Precise regulation of synthesis of extracellular matrix components such as collagen is obviously critical within all tissues. However it is of particular importance

in the lung since any deficiency of matrix components can cause alveolar airspace collapse and destruction, whilst any excess can distort and widen the pulmonary interstium thus preventing the close opposition of endothelial and epithelial monolayers. In both instances the capability of the lungs for efficient gaseous exchange will be seriously adversely affected.

1.1.5. Novel therapies for IPF

In a consensus statement of the American and European Thoracic Societies in 2000, prednisolone together with an immunosuppressant such as azathioprine was recommended as best standard care for IPF patients. In a small trial in 1991, a combination of azathioprine and prednisolone was shown to provide a survival advantage over prednisolone (Raghu et al. 1991), but surprisingly prednisolone has never been compared with placebo in a randomized controlled trial in terms of its effect on any meaningful physiological outcome or survival. This poses a major problem in the evaluation of novel therapies because due to the current recommendations on treatment of IPF, it is considered unethical to compare any novel therapy directly against placebo and withhold the use of prednisolone. Hence recent trials have effectively compared the novel therapy plus prednisolone against prednisolone alone. Recent phase III randomized placebo-controlled clinical trials of such design with interferon gamma-1b (IFN-γ-1b) (Raghu et al. 2004), pirfenidone (Azuma et al. 2005b), N-Acetylcysteine (Demedts et al. 2005), and anticoagulants (Kubo et al. 2005) have all shown limited but statistically significant benefits. It is hard to know whether these small benefits are clinically meaningful however. All of these agents have previously proved efficacious at attenuating experimentally-induced fibrosis in animal models (Hyde et al. 1988),(Iyer et al. 1995),(Shahzeidi et al. 1991),(Gunther et al. 2003),(Howell et al. 2001).

In a large trial of 330 patients there was a trend for a small survival benefit after 58 weeks of therapy (Raghu et al. 2004) with IFN-γ-1b, which became significant when only patients with early or mild disease were retrospectively considered (King, Jr. et al. 2005). IFN-γ-1b may exert antifibrotic effects by modulating the local Th_1/Th_2 cytokine balance in favour of a Th_1 profile, inhibiting

TGF-β signalling, and inhibiting fibroblast function and collagen synthesis (reviewed in (Thannickal et al. 2005)).

In a trial of 107 patients, pirfenidone reduced the rate of physiological decline and the number of exacerbations over a 12 month period (Azuma et al. 2005b). The antifibrotic influence of pirfenidone may be due to its ability to downregulate expression of PDGF and TGF-β_1, inhibit fibroblast activation and collagen synthesis, and function as an antioxidant (reviewed in (Thannickal et al. 2005)).

In a trial of 155 IPF patients over a 1 year period, N-Acetylcysteine significantly slowed the rate of physiological decline (Demedts et al. 2005). N-Acetylcysteine functions to replenish cellular stores of the antioxidant glutathione in the lung. This protects against injury mediated by reactive oxygen species and the potential consequent fibrosis.

In a limited trial of 56 patients over 3 years, anticoagulation demonstrated an impressive improvement in survival (Kubo et al. 2005). Anticoagulants function to inhibit generation of coagulation proteinases such as factor Xa and thrombin, and also the generation of fibrin. The potential pro-inflammatory and pro-fibrotic effects exerted by each of these will be discussed at length in a later section.

A number of other agents are also currently being evaluated as novel therapies for IPF, and these are outlined in **table 1.3**.

Novel therapeutic agent	Rationale for use
IFN-γ-1b	- Promotes Th_1 cytokine profile - Inhibits TGF-β signalling - Inhibits fibroblast function and collagen synthesis
Pirfenidone	- Downregulates PDGF and TGF-β_1 expression - Inhibits fibroblast activation and collagen synthesis - Antioxidant
N-Acetylcysteine	- Replenishes levels of the antioxidant glutathione in the lung. Thus protects against injury from reactive oxygen species
Anticoagulants	- Inhibits generation of FXa, Thrombin and fibrin, all of which are potentially pro-inflammatory and pro-fibrotic
Bosentan	- Endothelin receptor antagonist. Blocks endothelin-dependent activation of fibroblasts and collagen synthesis
Etanecept	- TNF-α antagonist. Blocks potentially pro-fibrotic effects of TNF-α
Imatinib mesylate (Gleevec)	- PDGF-receptor tyrosine kinase inhibitor. Blocks pro-fibrotic effects of PDGF. May also inhibit TGF-β_1 signalling.
Zileuton	- 5-lipoxygenase inhibitor. Inhibits production of pro-fibrotic Leukotrienes
Anti-CTGF antibodies	- blocks CTGF-dependent stimulation of fibroblast function
Tetrathiomolybdate	- Copper chelating compound. Downregulates the angigogenic factor VEGF (vascular endothelial growth factor) and pro-fibrotic factors such as the matricellular protein SPARC and TGF-β_1.
TGF-β_1 blocking strategies	- Monoclonal anti-TGF-β_1 antibodies - TGF-β_1 receptor kinase inhibitors - Inhibitors of TGF-β_1 signalling kinases - all designed to block profibrotic effects of TGF-β_1.

Table 1.3. Novel therapeutic agents currently being evaluated for treatment of IPF (adapted from (Thannickal et al. 2005))

1.1.6. Novel therapies for ARDS

As in IPF, a number of potential novel therapies are being evaluated, developed, or proposed, but none has yet made any impact on the considerable morbidity and mortality of ARDS. As mentioned above, treatment is largely supportive including oxygenation, protective ventilatory strategies, bronchodilators, inotropic support and optimization of fluid balance. Specific pharmacological therapies that have failed to show benefit in clinical trials to date in adult patients include N-Acetylcysteine, aerosolized surfactant, and the vasodilators nitroprusside, hydralazine, and prostacyclin (reviewed in (Ware & Matthay 2000)).

Steroids may confer some protection from fibrosing alveolitis in ARDS, and in a small randomized controlled trial of patients with unresolving ARDS, administration of methylprednisolone was associated with reduced bronchoalveolar lavage (BALF) type I and III procollagen aminoterminal propeptide levels, improved lung function, and improved survival (Meduri et al. 1998). A more recent trial has confirmed the beneficial effect of methylprednisolone, showing an improvement in pulmonary and extrapulmonary organ dysfunction, and reduced duration of mechanical ventilation and length of ICU stay (Meduri et al. 2007). However, a separate study suggests that the beneficial effects of steroids in ARDS might only be apparent in patients with co-existing adrenal insufficiency. Notably however this did include the majority of patients with septic shock-related ARDS examined (Annane et al. 2006). At present steroid use in ARDS remains controversial.

In contrast to IPF, ARDS is not often a single disease entity, but rather the pulmonary manifestation of the systemic inflammatory response syndrome. In fact, patients with ARDS often die of non-respiratory problems. Therapeutic strategies thus may need to be tailored to address the systemic- rather than just pulmonary dysfunction. In severe sepsis, which is commonly associated with ARDS, administration of recombinant APC reduces mortality by around 6% (Dhainaut et al. 2003), and this is likely to be mediated by systemic anti-inflammatory and anticoagulant effects. A trial examining the efficacy of APC specifically in ARDS, and led by Dr Michael Matthay in the USA is currently ongoing (personal communication, Dr Rachel Chambers).

1.1.7. Association of fibroproliferative lung disorders with excessive local procoagulant activity

Excessive procoagulant activity is a feature of a number of respiratory diseases which involve inflammation and deposition of extracellular matrix, including IPF (Chapman et al. 1986),(Ikeda et al. 1989),(Kotani et al. 1995), pulmonary fibrosis in systemic sclerosis (Ohba et al. 1994), ARDS (Bachofen & Weibel 1982), chronic lung disease of prematurity (Dik et al. 2003), cryptogenic organizing pneumonia (Peyrol et al. 1990), and airway remodelling in asthma (Gabazza et al. 1999). Work outlined in this thesis relates to the potential role of coagulation proteinases such as thrombin in the development of fibroproliferative lung disorders. The mechanisms by which coagulation occurs and the potential interplay between coagulation and disease processes will now be described in more depth.

1.2. The coagulation cascade and haemostasis

1.2.1. Coagulation pathways

Following vascular injury, an intricately controlled system involving platelets, plasma serine proteinases, cofactors, and inhibitors has evolved to effect the precise generation of thrombin, and subsequently fibrin. A fibrin clot enmeshed with platelets is fundamental in the restoration of integrity of the vessel wall, and thus prevents blood loss. The biochemical reactions involved in thrombin formation do not occur efficiently in solution, but rather require assembly of reactants and cofactors on phospholipid membranes of activated platelets and damaged cells in the presence of calcium ions. This effectively localizes clot formation to the site at which the process is initiated, and prevents unnecessary, and potentially disastrous, clotting occurring elsewhere in the vasculature.

Historically, formation of thrombin was thought to occur by two distinct pathways (**figure 1.1**) which converged in the activation of factor X to produce Factor Xa. Factor Xa then converts prothrombin to active thrombin. The extrinsic pathway was the pathway initiated by components outside of the circulation, and based on the observation by Morowitz in 1905 that introduction of tissue components into plasma culminated in the formation of fibrin. Conversely, the concept of the intrinsic pathway was based on the observation that blood placed in an artificial container clots without the addition of tissue "juices", suggesting coagulation could be initiated by components intrinsic to blood, and on contact. However it is now appreciated that these pathways are not independent. If that were so, then one might not have expected such a severe bleeding diathesis in patients with haemophilia A and B, who are deficient in clotting factors VIII and IX, respectively.

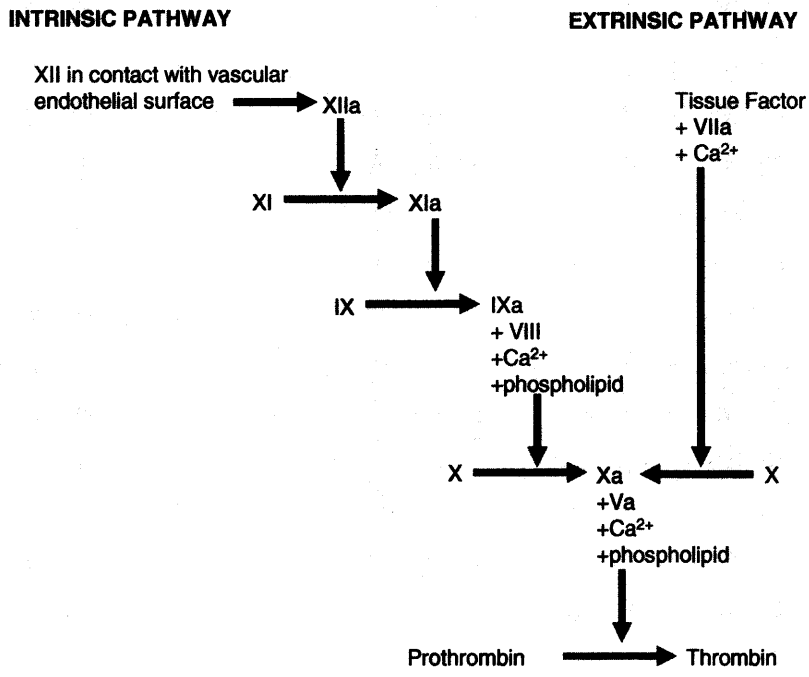


Figure 1.1. Old paradigm of thrombin generation.

Coagulation was thought to be initiated by either the intrinsic or extrinsic pathways, which converge in the activation of factor X to factor Xa, and the conversion of prothrombin to thrombin by factor Xa in conjunction with its cofactor Va. The intrinsic pathway was thought to be initiated when factor XII becomes activated on exposure to an endothelial surface. Subsequently, together with the accessory components prekallikrein and high molecular weight kininogen, factor XIIa is able to activate factor XIa. This leads to activation of IXa, which together with the cofactor VIIIa activates Xa. The extrinsic pathway was proposed to be initiated by circulating factor VII binding to extravascular tissue factor, and this complex is then able to activate Xa. Many of the reactions are dependent on calcium and require a phospholipid membrane surface. (Adapted from Oxford Handbook of Clinical Medicine, 2005, Longmore & Wilkinson (Eds)).

These factors were proposed to be necessary to activate factor X in the intrinsic pathway. However theoretically, factor X could have been activated independently of factors VIII and IX by the extrinsic pathway. Despite this, these patients have a profound inability to form effective blood clots. This suggested that the extrinsic pathway could not compensate for a defective intrinsic pathway, and that effective coagulation requires factors originally specifically ascribed to both pathways.

Current thinking regarding coagulation (reviewed in (Mann 2003) & (Hoffman 2003)) is illustrated in **figure 1.2.**

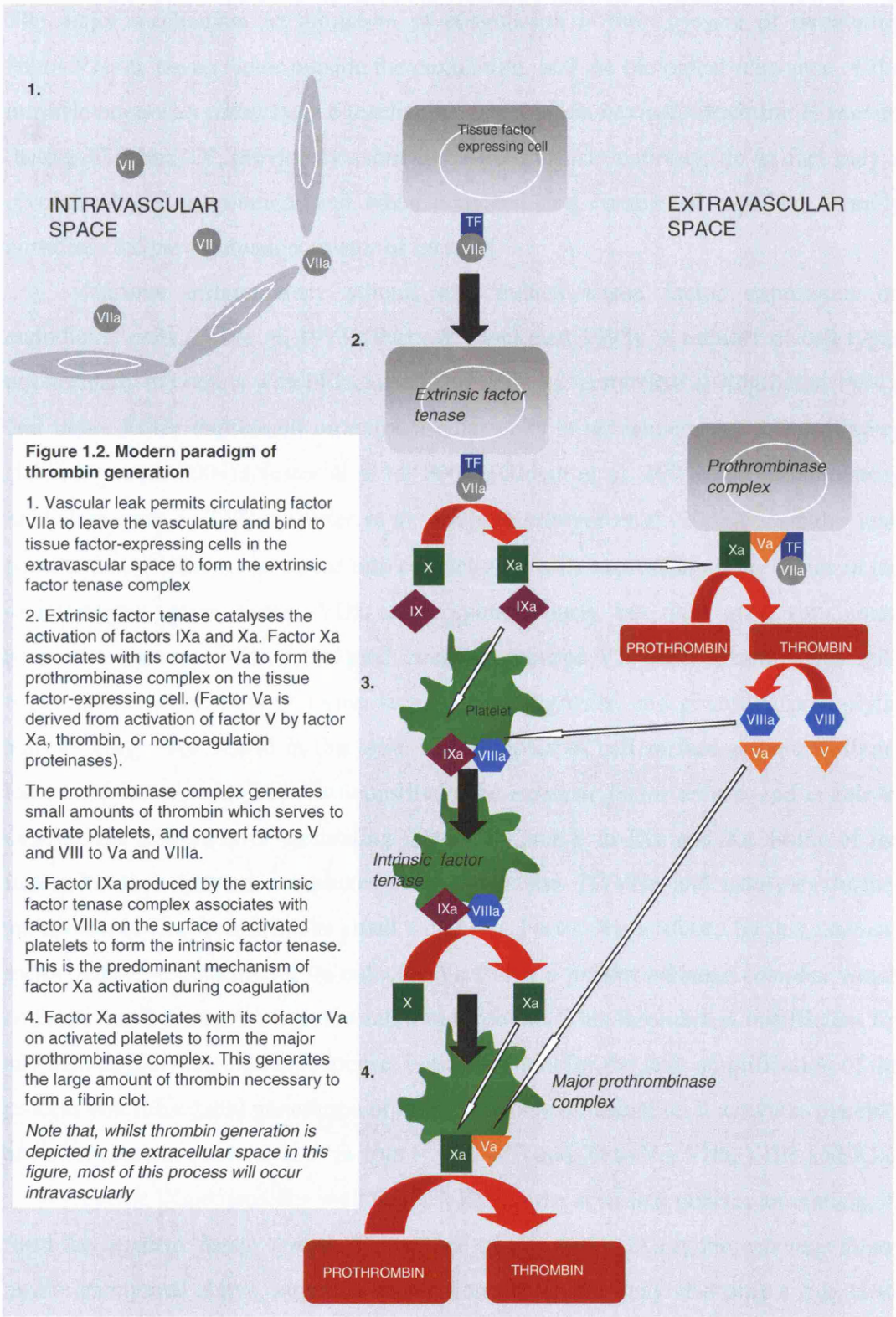


Figure 1.2. Modern paradigm of thrombin generation

1. Vascular leak permits circulating factor VIIa to leave the vasculature and bind to tissue factor-expressing cells in the extravascular space to form the extrinsic factor tenase complex

2. Extrinsic factor tenase catalyses the activation of factors IXa and Xa. Factor Xa associates with its cofactor Va to form the prothrombinase complex on the tissue factor-expressing cell. (Factor Va is derived from activation of factor V by factor Xa, thrombin, or non-coagulation proteinases).

The prothrombinase complex generates small amounts of thrombin which serves to activate platelets, and convert factors V and VIII to Va and VIIIa.

3. Factor IXa produced by the extrinsic factor tenase complex associates with factor VIIIa on the surface of activated platelets to form the intrinsic factor tenase. This is the predominant mechanism of factor Xa activation during coagulation

4. Factor Xa associates with its cofactor Va on activated platelets to form the major prothrombinase complex. This generates the large amount of thrombin necessary to form a fibrin clot.

Note that, whilst thrombin generation is depicted in the extracellular space in this figure, most of this process will occur intravascularly

The major mechanism for initiation of coagulation is the exposure of circulating factor VIIa to tissue factor outside the circulation, and the biological relevance of the intrinsic or contact pathway as a mechanism of initiation *in vivo* is doubtful. However, factors VIII and IX, previously ascribed to the intrinsic pathway, do in fact play a pivotal role in coagulation, and when activated and complexed together, actually constitute the predominant activator of factor X.

Various inflammatory stimuli can induce tissue factor expression on endothelial cells (Fei et al. 1993),(Parry & Mackman 1995). A number of cell types not normally in contact with blood also express tissue factor (Ruf & Edgington 1994); and tissue factor expression on extravascular cells is upregulated by cellular injury (Iakhiaev et al. 2004),(Nestoridi et al. 2005),(Olman et al. 1995) and inflammatory mediators such as TNF-α (Sitter et al. 1996),(Haslinger et al. 2003). Vascular leak permits factor VIIa to also come into contact with cells expressing tissue factor in the extravascular space. Factor VIIa exists spontaneously but only as a very small proportion (around 1%) of the total circulating factor VII pool. It complexes with tissue factor on surfaces of tissue factor-expressing cells, and possesses proteolytic activity only when bound in this way. The complex of cell surface-associated tissue factor and factor VIIa (TF/VIIa) constitutes the *extrinsic factor tenase*, and is able to catalyse the activation of circulating factors IX and X to IXa and Xa. Some of the factor Xa thus formed complexes with membrane TF/VIIa and catalyses further generation of IXa from IX. The small amount of Factor Xa produced by this *extrinsic factor tenase*, together with its cofactor Va forms a *prothrombinase complex* which converts small amounts of prothrombin to thrombin. This thrombin is insufficient for any meaningful coagulation response, but is essential for the later amplification of the process and subsequent generation of larger amounts of thrombin. It activates platelets, and catalyses the conversion of factors V VII, VIII and XI to Va, VIIa, VIIIa and XIa.

Factor IXa complexes with factor VIIIa on the activated platelet membrane, to form the *intrinsic factor tenase*. The source of this factor IXa is the *extrinsic factor tenase* mentioned above, although in addition, factor XIa may also play a role in its generation. Platelet activation occurs as a result of their exposure to either i) subendothelial collagen in an injured blood vessel, ii) thrombin, or iii) pro-inflammatory mediators such as platelet activating factor (PAF) (Zimmerman et al. 2002). The *intrinsic factor tenase* is far more efficient than the *extrinsic factor tenase*, and produces the bulk of the factor Xa involved in coagulation. Inability to assemble

the *intrinsic factor tenase* because of deficiency of factor VIII or IX is in fact the basis of the bleeding diathesis observed in patients with haemophilia. The large amounts of factor Xa produced combine with factor Va on the activated platelet membrane surface to form the *major prothrombinase complex*, which converts prothrombin to thrombin. Notably, this *prothrombinase complex* is the predominant site of conversion of prothrombin to thrombin. Thrombin catalyses the conversion of fibrinogen to fibrin and the release of fibrinopeptides A and B. This fibrin then polymerizes, is stabilized by Factor XIII-induced cross-linking, and entraps any activated adhered platelets at the initial site of vessel injury to form a stable blood clot.

1.2.2. Inhibitors of the coagulation cascade

Under normal conditions, the net effect of inhibitors of coagulation outweighs the potential coagulation response, such that the blood remains fluid and devoid of thromboses. The main inhibitors of coagulation are antithrombin, tissue factor pathway inhibitor (TFPI), and the thrombin-thrombomodulin protein C system. Antithrombin targets and neutralizes all the activated procoagulant serine proteinases, including thrombin. TFPI targets the extrinsic factor tenase, and prevents the generation of IXa, and Xa. The thrombin-thrombomodulin-protein C system is initiated when thrombin binds to thrombomodulin on the vascular endothelium. Thrombin then rapidly activates protein C, which is localised to the endothelial cell surface by the endothelial protein C receptor (EPCR). Following its subsequent release from the cell surface, activated protein C binds to circulating protein S, and together these proteins inactivate factors Va and VIIIa.

A number of therapeutic strategies are used to inhibit coagulation. Warfarin inhibits the vitamin K-dependent γ-carboxylation of glutamate residues, which is an integral process in the synthesis of coagulation proteinases VII, IX, X, and thrombin. Heparin binds to antithrombin, and increases its ability to inhibit thrombin, factor IXa, and factor Xa. Low molecular weight heparin also complexes with antithrombin but only increases its ability to inhibit Xa. Recombinant activated protein C has recently been successfully administered in sepsis (Dhainaut et al. 2003), although its efficacy in reducing mortality may be mediated via anti-inflammatory as well as anticoagulant actions (Macias et al. 2005).

1.2.3. Regulation of fibrin turnover

Fibrin turnover is regulated by a dynamic balance between factors that promote its removal and factors that inhibit this process. The expression and release of these factors are regulated in response to a number of injurious stimuli and inflammatory mediators. Plasmin, the active serine proteinase that degrades fibrin, is converted into its active form by tissue plasminogen activator (tPA) and urokinase (uPA), which are synthesized and secreted primarily by endothelial cells. Both tPA and uPA are inhibited by the widely expressed plasminogen activator inhibitor-1 (PAI-1). The activity of plasmin is regulated by a number of circulating inhibitors. Its generation is inhibited by thrombin activatable fibrinolysis inhibitor (TAFI), and its function is directly inhibited by α_2-plasmin inhibitor (α_2-PI) and α_2-macroglobulin (α_2-MG). TAFI also modifies carboxyterminal lysine residues of fibrin molecules rendering them less degradable by plasmin.

1.2.4. Crosstalk between coagulation and inflammation

Considerable cross-talk exists between coagulation and inflammation, and pro-coagulant and pro-inflammatory pathways interact at a number of levels. Activation of coagulation may serve a useful function in an inflammatory response, in that local deposition of fibrin forms a physical barrier to limit and contain the inflammatory response to the site of injury. However excessive activation of coagulation, as occurs in the context of a substantial systemic inflammatory response in sepsis results in disseminated intravascular coagulation which is itself responsible for severe organ damage.

An important point of interaction between inflammation and coagulation is the ability of inflammatory mediators to locally upregulate the expression of tissue factor, and hence promote the initiation of the coagulation cascade. In *in vitro* studies, tissue factor expression is increased in response to LPS, IL-1β, or TNF-α on endothelial cells (Fei et al. 1993),(Parry & Mackman 1995),(Pertynska-Marczewska et al. 2004), macrophages (Car et al. 1991),(Rashid et al. 1996), and vascular smooth muscle cells (Xuereb et al. 2000). IL-6 may also be important in upregulation of tissue factor, as *in vivo* studies have demonstrated that administration of anti-IL-6 antibodies attenuates

tissue factor-dependent thrombin generation in experimental endotoxaemia (van der et al. 1994). Inflammatory mediators such as LPS also stimulate release of platelet activating factor from macrophages (Leslie & Detty 1986) and thus promote platelet activation and thrombosis, whilst TNF-α can also impact on fibrin turnover. Injection of TNF-α into healthy volunteers induces a rapid transient increase in fibrinolytic activity associated with increased tPA and uPA levels, but a subsequent decrease in fibrinolytic activity associated with increased PAI-1 levels (van der et al. 1991). As well as promoting coagulation, inflammation can inhibit physiological anticoagulant mechanisms. For instance, levels of antithrombin and protein C are reduced in sepsis. This may relate to reduced synthesis, but in addition these proteins may be degraded by neutrophilic proteinases, since neutrophil elastase has been shown to degrade both of these mediators *in vitro* (Esmon & Yee 1992),(Eckle et al. 1991).

Conversely coagulation proteinases can influence inflammation via signalling through specific cellular receptors, known as proteinase activated receptors (PARs). These receptors will be discussed in detail in the following section: **1.3. Proteinase activated receptors**. Briefly, activation of PAR_1, the major cellular receptor for thrombin, promotes inflammation by increasing vascular endothelial cell permeability (Rabiet et al. 1996), upregulating endothelial adhesion molecule expression (Sugama et al. 1992), and stimulating the release of pro-inflammatory mediators from a variety of cell types (Colotta et al. 1994),(Johnson et al. 1998),(Ueno et al. 1996),(Naldini et al. 2000). Similarly Factor Xa has been shown to be capable of inducing the production of a range of pro-inflammatory mediators via activation of PAR_1 (Bachli et al. 2003) and PAR_2 (Papapetropoulos et al. 1998), (Lee et al. 2005b) depending on cell type.

Fibrinogen, the circulating precursor of fibrin, may also promote inflammation. *In vitro* studies have demonstrated that fibrinogen can induce the expression and release of a host of pro-inflammatory mediators from neutrophils (Walzog et al. 1999), macrophages (Smiley et al. 2001), endothelial cells (Qi & Kreutzer 1995) and fibroblasts (Liu & Piela-Smith 2000). Fibrin also serves as an important matrix to which inflammatory cells can adhere. In experiments involving implantation of synthetic biomaterials into the peritoneal cavities of mice, the inflammatory response was found to be fibrinogen-dependent in that fibrinogen-deficient mice failed to

mount an inflammatory response unless the biomaterial was first precoated with fibrinogen (Tang & Eaton 1993).

Activated protein C (APC) is an endogenous anticoagulant which, in conjunction with protein S, inactivates factors Va and VIIIa. However, in addition to this role as a physiological anticoagulant, it has anti-inflammatory effects. These occur following its binding to EPCR and are mediated via activation of PAR_1 (Riewald et al. 2002). In *in vitro* studies, APC attenuates endotoxin-induced production of TNF-α by monocytes (Yuksel et al. 2002). In animal studies, following exposure to endotoxin, administration of APC inhibits pulmonary leukocyte accumulation (Murakami et al. 1996), production of TNF-α and nitric oxide (NO), and hypotension (Isobe et al. 2001). Such an anti-inflammatory effect together with an anticoagulant effect may be the basis for the ability of APC to reduce mortality by 6% in patients with severe sepsis (Dhainaut et al. 2003).

1.2.5. Crosstalk between coagulation, fibrin turnover, and fibrosis

Cross talk also exists between coagulation and fibrosis at a number of levels. Coagulation proteinases exert a number of pro-fibrotic effects. For example thrombin and factor Xa activate PAR_1 to promote fibroblast mutagenesis and synthesis of extracellular matrix proteins such as collagen (Hernandez-Rodriguez et al. 1995),(Chambers et al. 1998),(Blanc-Brude et al. 2005). Thrombin similarly induces production of the pro-fibrotic growth factors PDGF, CTGF, and TGF-β_1 (Shimizu et al. 2000),(Chambers et al. 2000),(Bachhuber et al. 1997) from a number of cell types. Conversely, as well as being anti-inflammatory, the anticoagulant APC may exert anti-fibrotic effects. For example, APC has been shown to inhibit thrombin-induced PDGF expression in epithelial cells and macrophages *in vitro*, and to reduce lung PDGF expression to block lung fibrosis in response to bleomycin in mice (Shimizu et al. 2003).

The fibrotic environment may impact on coagulation and fibrin turnover. Mediators implicated in fibrogenesis such as the growth factors PDGF and TGF-β_1, and the CC chemokine CCL2 have been shown to induce tissue factor expression *in vitro* (Xuereb et al. 1997),(Ernofsson & Siegbahn 1996),(Ranganathan et al. 1991), and thus may promote the initiation of coagulation. The fibrotic environment may also

favour fibrin persistence. Constitutive PAI-1 expression is increased in fibroblasts isolated from fibrotic tissue (Tuan et al. 1996),(Yang et al. 2003), and TGF-β_1 has been shown to increase PAI-1 expression by numerous cell types including tracheal epithelial cells *in vitro* (Idell et al. 1994).

Thus coagulation exerts powerful influences on inflammation and fibrosis, and these processes themselves have a profound effect on coagulation. Excessive procoagulant activity is a feature of a number of respiratory diseases which involve inflammation and deposition of extracellular matrix. Hence crosstalk between coagulation, inflammation and fibrosis may play an important part in the pathogenesis of these conditions.

1.3. Proteinase activated receptors (PARs)

As alluded to previously, coagulation proteinases are able to activate a number of specific cellular receptors, known as proteinase activated receptors (PARs), to exert a host of cellular effects. Importantly many of these effects are distinct from the role of coagulation proteinases in haemostasis, although activation of PAR_1, PAR_3, and PAR_4 by thrombin contributes to activation of human and mouse platelets, and so clearly *is* relevant to haemostasis.

PARs are a family of seven-transmembrane G-protein coupled receptors. The family consists of four members, designated $PARs_{1-4}$. A number of proteinases besides those associated with coagulation serve as agonists for PARs. The receptors can also be activated by small specific peptide agonists which correspond to the activating tethered ligand sequence intrinsic to each receptor. The agonists for each receptor, together with tissue localization of the receptors, and their potential physiological roles are shown in **table 1.4**.

Receptor	Physiological agonists	Agonist peptide	Tissue localization	Known/ potential physiological role
PAR_1	Thrombin Factor Xa TF-VIIa-Xa APC Plasmin	SFLLRN TFLLRN	Brain, lung, heart, stomach, colon, kidney, testis	Human platelet activation, regulation of vascular tone, modulator of nociception, embryonic development, pro-inflammatory, pro-fibrotic
PAR_2	Factor Xa TF-VIIa-Xa Trypsin Tryptase	SLIGKV	Prostate, small intestine, colon, liver, kidney, pancreas, trachea	Regulation of vascular tone, mediator of nociception, airway protection, pro-inflammatory
PAR_3	Thrombin	None	Heart, kidney, pancreas, thymus, small intestine, stomach, lymph node, trachea	Co-factor for PAR_4, mouse platelet activation
PAR_4	Thrombin Trypsin Cathepsin G	GYPGQV AYPGKF	Lung, pancreas, thyroid, testis, small intestine, placenta, skeletal muscle, lymph node, adrenal gland, prostate, uterus, colon	Platelet activation

Table 1.4. PARs, their agonists, tissue localization, and potential physiological role
(Adapted from (Ossovskaya & Bunnett 2004),(Hollenberg & Compton 2002),(Hollenberg 2003) & (Chambers & Laurent 2002))

1.3.1. Mechanism of activation of PARs

PARs display a unique mechanism of activation in that they are not activated by a classical circulating ligand but rather by their own intrinsic tethered ligand. Their agonist serine proteinase recognizes and specifically interacts with a peptide sequence near the receptor N-terminus and cleaves a portion off the receptor at a specific site. This effectively unmasks a previously anchored tethered ligand and allows it to bind intramolecularly to the second extracellular loop of the receptor. This interaction produces a conformational change at the receptor's intracellular C-terminus allowing it to interact with membrane associated heterotrimeric G-proteins. An exchange of GTP for GDP and the dissociation of α and $\beta\gamma$ G-protein subunits follows, and subsequently these subunits initiate cell signalling via specific pathways. Signalling responses following activation of $PARs_{1,3 \text{ and } 4}$ are each associated with a rapid increase in intracellular calcium, which is easily measurable *in vitro*, and most likely occurs downstream of receptor coupling to a $G\alpha_q$ G-protein subunit. The coupling of PARs to distinct G-protein subunits dictates the nature of the cellular response and depends on the type of PAR, and also probably the cell type and relative local abundance of different G-proteins (reviewed in (Hollenberg & Compton 2002)). The mechanism of activation of PARs is illustrated in **figure 1.3.**

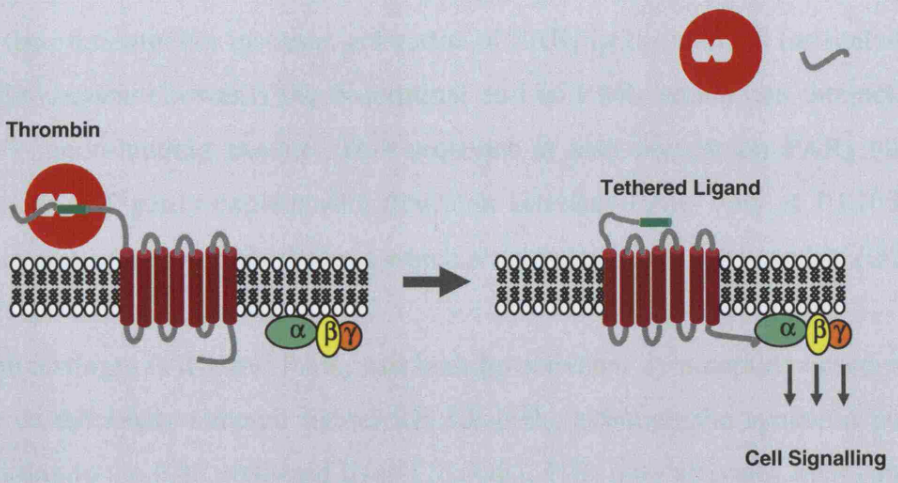


Figure 1.3. Mechanism of PAR activation. In this figure, activation of PAR_1 by thrombin is illustrated. The N terminus of the receptor is recognised and cleaved by thrombin. This liberates a 41 amino-acid fragment and unmasks a tethered ligand which binds intramolecularly to the 2nd extracellular loop of the receptor. The resulting receptor conformational change allows the C terminus to interact with membrane-associated G proteins, and initiate cell signalling.

Following activation of a PAR, its activating proteinase retains its ability to activate other similar PARs on the cell surface, such that in theory a low concentration of proteinase could cleave the same amount of PAR as a high concentration, but over a longer duration. Thus the conventional means of achieving graded cellular responses to differing agonist concentrations by differing degrees of receptor occupancy is not possible for PARs. Instead, graded responses in PARs are achieved by sensing the rate of generation of intracellular signalling mediators such as IP_3. A fast rate of generation of these mediators correlates with a high extracellular agonist proteinase concentration.

A further unique feature of PARs is that they can be activated experimentally by short synthetic peptides with sequences analogous to those of their intrinsic tethered ligand, for instance PAR_1 can be activated by SFLLR-NH_2 or TFLLR-NH_2 peptides. However, these receptor activating peptides are generally less efficient than the *in vivo* physiological activators of the PARs because they do not enable certain

specific interactions to occur between receptor and proteinase which are important to facilitate their binding. For instance, activation of PAR_1 by thrombin is facilitated by a hirudin-like sequence towards the N-terminal end of PAR_1 which can interact with thrombin's anion-binding exosite. This sequence is also present on PAR_3 but not PAR_4, which may partly explain why thrombin activates PAR_4 only at 10-100 fold higher fold concentrations that those at which it activates PAR_1 (reviewed in (O'Brien et al. 2001)).

Interestingly PAR_1 and PAR_2 can both be activated by a peptide based on the sequence of the PAR_1 tethered ligand SFLLR-NH_2, although the synthetic peptide corresponding to the PAR_2 tethered ligand SLIGRL-NH_2 only activates PAR_2 and not PAR_1. This suggests that intermolecular activation between PAR_1 and PAR_2 might occur physiologically following proteolytic unmasking of the PAR_1 tethered ligand. In support of this, O'Brien et al (O'Brien et al. 2000) showed that transfection of COS7 cells with both wild-type PAR_2 and a PAR_1 mutant, which could be cleaved but could not signal, was associated with a response to thrombin not seen when PAR_2 alone was transfected. It also had important implications for experimental design, since responses to the SFLLR-NH_2 peptide cannot be reliably attributed to PAR_1 activation. Subsequent structure activity studies identified that the TFLLR-NH_2 peptide was selectively able to activate PAR_1 but not PAR_2. This is the peptide utilised for *in vitro* experiments in this thesis, and the partial reverse peptide FTLLR-NH_2 is used as a control peptide to confirm the specificity of its responses.

PAR_3 is unusual in that it is not capable itself of generating an intracellular signal, but rather serves to function as a cofactor for PAR_4 cleavage. Experiments involving expression of mouse PAR_3 ($mPAR_3$) and/or $mPAR_4$ in COS7 cells demonstrated that co-expression of $mPAR_3$ and $mPAR_4$ rendered cells responsive to thrombin at a far lower EC_{50} than if $mPAR_4$ was expressed alone, whilst expression of $mPAR_3$ alone did not render cells responsive to thrombin at all. Furthermore the rate of thrombin-induced $mPAR_4$ cleavage was far greater when $mPAR_3$ and $mPAR_4$ were co-expressed than when $mPAR_4$ alone was expressed (Nakanishi-Matsui et al. 2000). Nakanishi-Matsui *et al* (Nakanishi-Matsui et al. 2000) propose that thrombin first binds and cleaves PAR_3, but then remains tethered via PAR_3's hirudin-like sequence, and then cleaves and activates PAR_4. Notably this cofactor action of PAR_3 entirely distinct from the transactivation of PAR_2 by PAR_1 described above. However it is possible that PAR_3 is additionally capable of receptor transactivation since peptides

corresponding to PAR_3-derived tethered ligand sequences have been shown to be capable of activating $PARs_{1 \text{ and } 2}$ in Jurkat T cells (Hansen et al. 2004).

Interestingly the requirement for PARs in the activation of platelets is distinct in mice and humans. In humans, platelet activation at low concentrations of thrombin is mediated by PAR_1 and at high concentrations also by PAR_4. PAR_3 is unlikely to contribute since RT-PCR analysis has demonstrated its absence at the mRNA level (Kahn et al. 1999). In mice, plaletet activation is not dependent on PAR_1 since platelets from $PAR_1^{-/-}$ mice exhibit the same responsiveness to thrombin as those from wild-type mice (Connolly et al. 1996). Mouse platelets express PAR_3 and PAR_4, and at low concentrations of thrombin, PAR_3 supports cleavage of PAR_4 as mentioned previously (Kahn et al. 1998b).

1.3.2. Regulation of PAR signalling (reviewed in (Dery et al. 1998) & (Hollenberg & Compton 2002))

A number of mechanisms may contribute to the cessation of signalling by PARs. Proteinases are likely to diffuse away from the vicinity of their receptors, and may be subject to degradation by proteinase inhibitors. However the potential presence of an activating tethered ligand permanently attached to the receptor necessitates physiological mechanisms for PARs to be rendered inactive.

1.3.2.1 Receptor deactivation

PARs may be subject to proteolytic inactivation. For instance, the proteinases neutrophil elastase, cathepsin G and proteinase 3 can each inactivate PAR_1 by cleaving the receptor at a specific site C-terminal to its tethered ligand, thus amputating the ligand from the receptor body. These deactivated receptors are subsequently refractory to activation by proteinases but still responsive to experimental activation by synthetic peptides. (Renesto et al. 1997). Proteinase targeting of the tethered ligand once it is bound to the receptor's second extracellular loop has also been demonstrated *in vitro* with thermolysin, and may represent a further mechanism of deactivation *in vivo* (Chen et al. 1996).

1.3.2.2. Receptor desensitization

Receptor desensitization involves the uncoupling of PARs from G proteins and their signalling pathways. Homologous receptor desensitization involves agonist-dependent phosphorylation of specific C-terminal residues of the receptor by G protein receptor kinases, which utilise proteins known as β-arrestins as cofactors. This receptor phosphorylation effectively disrupts receptor interaction with G proteins and halts signal transduction. Heterologous receptor desensitization can occur as a result of C-terminal phosphorylation by other intracellular protein kinases such as protein kinases C and A, and may provide a means whereby PAR responses are influenced by other signalling pathways.

1.3.2.3. PAR endocytosis and cycling

A further means of regulating responsiveness of PARs is their endocytosis following activation. This process is also dependent on specific residues at the receptor's C-terminal end. Following activation, cleaved receptors are rapidly internalized in clathrin coated pits (Hoxie et al. 1993). Some are then degraded in lysozomes, whilst some traffic back to the cell surface but remain unresponsive to further proteolytic activation (Brass et al. 1994). Receptor endocytosis also occurs independently of receptor activation, and PAR_1 has been shown to cycle constitutively between the plasma membrane and intracellular stores (Shapiro et al. 1996).

1.3.2.4. Restoration of cellular responses

The ability of a cell to regain responsiveness to PAR activation depends on both the mobilization of intracellular receptor stores and the synthesis of new receptors. Abundant intracellular stores of PARs have been reported in endothelial cells (Horvat & Palade 1995), fibroblasts (Hein et al. 1994), and epithelial cells (Kong et al. 1997); although in all cell types recovery of responses after prolonged exposure to activating proteinases depends on protein synthesis of new receptors. Platelets lack the ability to synthesize new receptors and have limited intracellular receptor pools. Thus they are unable to regain responsiveness following their activation by thrombin.

1.3.2.5. PAR downregulation

Unlike receptor desensitization, which involves uncoupling of the receptor from its G proteins, receptor downregulation involves an active reduction in the total amount of receptor expressed by the cell. Downregulation may de due to decreased receptor synthesis or to increased receptor degradation. In mesangial cells exposed to thrombin for prolonged periods, downregulation is associated with reduced PAR_1 cellular protein but unchanged PAR_1 mRNA, consistent with a mechanism involving increased receptor degradation (Zacharias et al. 1995). As such downregulation occurs in response to the receptors agonist proteinase it is termed homologous downregulation. In contrast, in these same cells, a heterologous downregulation of PAR_1 can occur as a result of inhibition of PAR_1 gene expression by PGE_1 activation of adenylylcylase.

1.4. Proteinase-Activated Receptor-1 (PAR_1)

PAR_1 is the major cellular receptor for thrombin, although it can also be activated by factor Xa, ternary complexes of Tissue Factor with VIIa and Xa (Riewald et al. 2001), activated protein C (Riewald et al. 2002), and plasmin (Mandal et al. 2005). As mentioned previously the efficiency of thrombin's activation of PAR_1 is facilitated by a hirudin-like binding domain C-terminal to the tethered ligand at PAR_1's N-terminus. The attraction between this domain and thrombin's anion binding exosite enables thrombin to effectively complex with PAR_1, whereupon it recognizes and cleaves the receptor immediately distal to its tethered ligand. Efficient activation of PAR_1 by factor Xa is facilitated by localization of factor Xa to the cell surface. Two mechanisms make this possible. Firstly, it can remain associated with tissue-factor and factor VIIa at the site of its generation. Secondly it can bind to effector-cell protease receptor-1 (EPR-1). Factor Xa is more effective at activating PAR_1 as part of either of these conformations than as factor Xa alone (Riewald et al. 2001),(Blanc-Brude et al. 2001). Similarly, APC utilizes cell surface EPCR as a cofactor to facilitate its activation of PAR_1 (Riewald et al. 2002).

The PAR_1 gene has a similar structure to the other PARs and comprises a single large intron interrupting two exons at the region encoding the receptor's N terminus. In humans PAR_1 is located in a cluster with PAR_2 and PAR_3 at chromosome 5q13, and PAR_4 is located separately at 19p12. In mice, $PARs_{1-3}$ are clustered at 13d2 and PAR_4 is at 8b3 (Kahn et al. 1998a),(Xu et al. 1998). A number of PAR_1 polymorphisms have been found to influence platelet receptor density (Dupont et al. 2003) and thrombosis-related events (Smith et al. 2005),(Arnaud et al. 2000), but to date none have been found to have any association with inflammatory or fibrotic diseases. The generation of a $PAR_1^{-/-}$ mouse by Connolly et al in 1996 (Connolly et al. 1996) has been of enormous value in furthering our understanding of PAR_1 biology. This mouse was generated by inserting a neocassette to displace exon 2 of the PAR_1 gene. This strategy was effective since this large exon encodes the majority of the receptor from its intracellular C terminus to part of its N-terminus. The phenotype of the $PAR_1^{-/-}$ mouse is grossly normal, although there is a 50% intraembryonic lethality due to a defect in vascular development (Griffin et al. 2001), implicating a role for PAR_1 in endothelial function in developing blood vessels. In previous studies, $PAR_1^{-/-}$

mice have exhibited protection from experimentally induced glomerulonephritis (Cunningham et al. 2000), arthritis (Yang et al. 2005), and cerebral infarction (Junge et al. 2003). This clearly supports the role of PAR_1 in mediating responses to tissue injury. In work outlined in this thesis, $PAR_1^{-/-}$ mice were utilized to evaluate the potential role of PAR_1 in mediating responses to injury within the lung.

Following activation, PAR_1 exerts a multitude of diverse cellular responses. The diversity of these responses results from PAR_1 coupling to different G protein subunits and their downstream signalling pathways. Major pathways are illustrated in **figure 1.4**. In the same cell type responses to different agonists may vary. For example thrombin and the PAR_1 agonist peptide TFLLR-NH_2 both activate PAR_1 on endothelial cells to influence barrier permeability and calcium mobilization. However activation by thrombin is associated with more efficient receptor coupling to $G\alpha_{12/13}$ and a greater effect on barrier permeability, whereas activation by TFLLR-NH_2 is associated with more efficient receptor coupling to $G\alpha_q$ and a greater effect on calcium mobilization. (McLaughlin et al. 2005b). This phenomenon is called functional selectivity, and may be a potential explanation for the distinct gene expression profiles observed in cytokine-stimulated endothelial cells in response to activation of PAR_1 by APC and thrombin (Riewald & Ruf 2005).

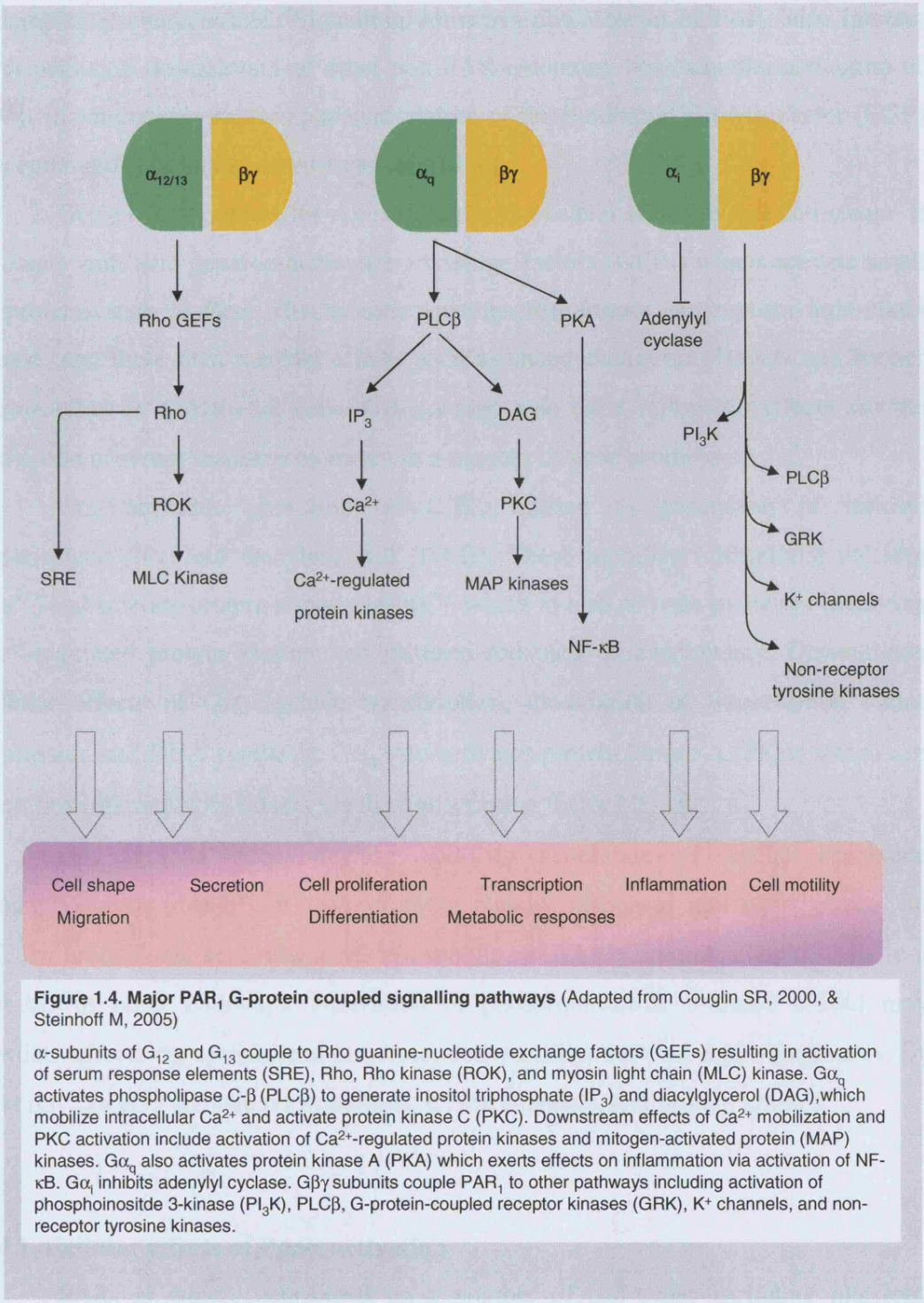


Figure 1.4. Major PAR_1 G-protein coupled signalling pathways (Adapted from Couglin SR, 2000, & Steinhoff M, 2005)

α-subunits of G_{12} and G_{13} couple to Rho guanine-nucleotide exchange factors (GEFs) resulting in activation of serum response elements (SRE), Rho, Rho kinase (ROK), and myosin light chain (MLC) kinase. $G\alpha_q$ activates phospholipase C-β (PLCβ) to generate inositol triphosphate (IP_3) and diacylglycerol (DAG),which mobilize intracellular Ca^{2+} and activate protein kinase C (PKC). Downstream effects of Ca^{2+} mobilization and PKC activation include activation of Ca^{2+}-regulated protein kinases and mitogen-activated protein (MAP) kinases. $G\alpha_q$ also activates protein kinase A (PKA) which exerts effects on inflammation via activation of NF-κB. $G\alpha_i$ inhibits adenylyl cyclase. Gβγ subunits couple PAR_1 to other pathways including activation of phosphoinositde 3-kinase (PI_3K), PLCβ, G-protein-coupled receptor kinases (GRK), K^+ channels, and non-receptor tyrosine kinases.

PAR_1 couples to α_q, $\alpha_{12/13}$, α_i, and βγ G-protein subunits to initiate specific cell signalling pathways (reviewed in (Ossovskaya & Bunnett 2004), (Dery et al. 1998), (Coughlin 2000), (Steinhoff et al. 2005)). These cell signalling pathways involve multiple interactions at a number of levels, and even to this day are

incompletely characterized. Signalling pathways downstream of PAR_1 also interact with pathways downstream of other non-PAR receptors. For example, activation of PAR_1 in enterocytes induces phosphorylation of the Epidermal Growth Factor (EGF) Receptor and subsequent downstream signalling.

$G\alpha_{12/13}$ is important for regulation of cytoskeletal structure and cell shape. It interacts with Rho guanine-nucleotide exchange factors (GEFs) which activate small G proteins such as Rho. This in turn activates Rho-kinase and myosin light-chain kinase, and these then mediate effects such as shape change in platelets and barrier permeability in endothelial cells. $G\alpha_{12/13}$ may also exert mitogenic effects via the activation of serum response elements in a number of gene promoters.

$G\alpha_q$ activates phospholipase C-β_1, leading to generation of inosityl triphosphate (IP_3) and diacylglycerol (DAG). These mobilize intracellular calcium (Ca^{2+}) and activate protein kinase C (PKC), which in turn activate pathways involving Ca^{2+}-regulated protein kinases and mitogen-activated protein kinases. Downstream cellular effects of $G\alpha_q$ include transcription, modulation of transcription factor expression and DNA synthesis. $G\alpha_q$ also activates protein kinase A (PKA) which can exert pro-inflammatory effects via the transcription factor NF-κB.

$G\alpha_i$ inhibits adenylylcylase and its generation of cyclic adenosine monophosphate (cAMP). It may promote platelet responses and exert effects on cellular arachidonic acid release via phospholipase A2. βγ subunits couple PAR_1 to a number of other pathways. Activation of phosphoinositide 3-kinase (PI_3K) may mediate effects on cytoskeletal structure, cell motility, survival and mitogenesis. βγ subunits can also activate phospholipase C, ion channels, and protein kinases.

1.4.1. Cellular effects of PAR_1 activation

PAR_1 is widely distributed on a number of cell types including platelets, endothelial cells, fibroblasts, epithelial cells, monocytes, and neuronal cells. A pathological role of PAR_1 is suggested by increased local expression in disease entities involving injury of the synovium (Morris et al. 1996), kidney (Grandaliano et al. 2001), liver (Marra et al. 1998), and lung (Bogatkevich et al. 2005). This increased expression may result from exposure to pro-inflammatory and pro-fibrotic mediators

such and IL-1b, TNF-α and TGF-β_1 (Mbebi et al. 2001). A number of the cellular effects of PAR_1 activation outlined below may be pathological in the disease setting.

1.4.1.1. Platelet aggregation

In humans, thrombin induces platelet activation via PAR_1 and PAR_4, although this occurs primarily via PAR_1 at low thrombin concentrations. Thrombin also influences platelet aggregation independently of PARs via binding to platelet membrane glycoprotein GP1βα. Thrombin-activated platelets change shape and release serotonin, adenosine triphosphate, and thromboxane A2. P-selectin and CD40 ligand are also translocated to the platelet membrane which promotes their binding to endothelial cells (Stenberg et al. 1985), (Henn et al. 1998). Conversely as mentioned previously, platelet activation in mice is PAR_1 independent, and is instead mediated by PAR_3 and PAR_4.

1.4.1.2. Endothelial function

PAR_1 activation on endothelial cells by thrombin promotes expression of tissue factor (Bartha et al. 1993), and adhesion molecules such as E and P-selectin, and ICAM-1 (Sugama et al. 1992),(Shankar et al. 1994). This promotes initiation of coagulation and platelet adhesion, but also mediates trafficking of inflammatory cells to sites of injury. Thrombin also increases endothelial vascular permeability via change in cell shape by effects on the cytoskeleton (Siflinger-Birnboim & Johnson 2003), (McLaughlin et al. 2005b) and via the release of histamine from mast cells (Cirino et al. 1996). Interestingly, activation of PAR_1 on the endothelium by APC is associated with cellular effects that are distinct to those that occur in response to thrombin. APC reduces rather than enhances endothelial barrier permeability (Feistritzer & Riewald 2005). Furthermore i*n vitro* gene expression profiling studies using cytokine-perturbed human endothelial cells, thus representing the endothelium in sepsis, have revealed a number of genes that are differentially regulated in response to thrombin and APC. In these studies thrombin induced a number of genes associated with inflammation, such as IL-6 and IL-8, whereas APC induced far fewer genes but

suppressed expression of inflammatory genes such as NF-κB family members, and proapoptotic genes such as p53 (Riewald & Ruf 2005).

1.4.1.3. Vascular tone

In the vasculature, activation of PAR_1 on endothelial cells and underlying smooth muscle cells can affect vascular tone. Thrombin induces relaxation of smooth muscle via the release of nitric oxide from endothelial cells (Derkach et al. 2000). Conversely it induces strong contractile responses in smooth muscle in blood vessels from which the endothelial layer has been removed (Ku & Zaleski 1993). It is probable that the relative expression of PAR_1 on endothelial and smooth muscle layers may govern the resulting vascular tone and blood flow in any organ. Notably however, the observation that PAR_1-deficient mice have normal cardiac function and blood flow suggests that PAR_1 is only one of many influences in this regard (Darrow et al. 1996).

1.4.1.4. Cell growth, proliferation and survival

Thrombin promotes proliferation of mesenchymal cell types including fibroblasts, vascular smooth muscle cells, and endothelial cells. These mitogenic effects are also apparent in response to PAR_1 activating peptides (Strukova et al. 2001),(McNamara et al. 1993),(Herbert et al. 1994), and additionally in response to factor Xa in fibroblasts, and may be dependent on secondary mediators such as platelet derived growth factor (PDGF) (Blanc-Brude et al. 2001). PAR_1 activation also influences turnover of extracellular matrix components, and thrombin stimulates procollagen synthesis in fibroblasts and smooth muscle cells (Chambers et al. 1998), (Dabbagh et al. 1998). Interestingly, PAR_1 activation can apparently promote both cell survival and cell death. It promotes survival of neurones exposed to β-amyloid (Pike et al. 1996), but promotes apoptosis and increased epithelial permeability of intestinal epithelial cells (Chin et al. 2003).

1.4.1.5. Pro-inflammatory and pro-fibrotic effects

As described above, PAR_1 activation can promote inflammation by influencing inflammatory cell trafficking via upregulation of endothelial adhesion molecule expression. Its ability to promote mitogenesis and extracellular matrix protein synthesis in fibroblasts are central to its pro-fibrotic role. PAR_1 activation has also been reported to be capable of inducing apoptosis of lung epithelial cells (Suzuki et al. 2005). Epithelial cell apoptosis is implicated as promoting fibrosis and its inhibition attenuates the fibrotic response to bleomycin in rats (Wang et al. 2000b). The basis of this effect may be a loss of the inhibitory influence exerted by epithelial cells on lung fibroblast proliferation.

Importantly, PAR_1 activation may promote both inflammation and fibrosis by the generation of secondary mediators from a variety of cell types, and there is a wealth of published reports documenting this. **Table 1.5** lists examples of pro-inflammatory and pro-fibrotic secondary mediators released in response to PAR_1 activation and their documented cellular source. Pro-inflammatory mediators include the chemokines RANTES and IL-8; the cytokines TNF-α, IL-1 and IL-8; and COX-2: the enzyme that is necessary for synthesis of pro-inflammatory prostanoids. Pro-fibrotic mediators include the growth factors PDGF, TGF-β_1, CTGF, and bFGF which promote fibroblast proliferation, migration, and/or extracellular matrix protein synthesis; and thrombospondin-1, which may exert a profibrotic influence via its ability to activate TGF-β_1. CCL2 and ET-1 exert both pro-inflammatory and pro-fibrotic effects. CCL2 induces mononuclear cell and eosinophil chemotaxis, but also promotes fibroblast function. ET-1 enhances release of proteinases and cytokines from inflammatory cells, but also promotes fibroblast chemotaxis and proliferation.

Pro-inflammatory mediators	**Cellular source**
RANTES	Synovial fibroblasts (Hirano et al. 2002)
CCL2	Monocytes (Colotta et al. 1994) Endothelial cells (Colotta et al. 1994),(Marin et al. 2001) Ovarian luteinized granulosa cells (Hirota et al. 2003) Endometrial stromal cells (Hirota et al. 2005) Dermal fibroblasts (Bachli et al. 2003) Hepatic stellate cells (Fiorucci et al. 2004)
TNF-α	Glial cells (Lee et al. 2005a)
IL-1	Monocytes (Naldini et al. 2000)
IL-6	Monocytes (Naldini et al. 2000) Retinal epithelial cells (Scholz et al. 2004) Lung epithelial cells (Asokananthan et al. 2002)
IL-8	Endothelial cells (Marin et al. 2001) Retinal epithelial cells (Scholz et al. 2004) Lung epithelial cells (Asokananthan et al. 2002) Ovarian luteinized granulosa cells (Hirota et al. 2003) Endometrial stromal cells (Hirota et al. 2005) Dermal fibroblasts (Bachli et al. 2003)
COX2	Endothelial cells (Houliston et al. 2002) Endometrial stromal cells (Hirota et al. 2005) Colonic fibroblasts (Seymour et al. 2003)
Pro-fibrotic mediators	**Cellular source**
PDGF	Alveolar macrophages (Tani et al. 1997) Endothelial cells (Shankar et al. 1994) Lung fibroblasts (Blanc-Brude et al. 2001) Vascular smooth muscle cells (Stouffer & Runge 1998) Lung epithelial cells (Shimizu et al. 2000)
CTGF	Lung fibroblasts (Chambers et al. 2000)
TGF-β_1	Vascular smooth muscle cells (Stouffer & Runge 1998)
bFGF	Vascular smooth muscle cells (Stouffer & Runge 1998)
ET-1	Endothelial cells (Eto et al. 2001)
TSP-1	Endothelial cells (McLaughlin et al. 2005a)

Table 1.5. Pro-inflammatory and pro-fibrotic mediators induced in response to PAR_1 activation

During the course of this thesis, lung levels of the $PAR\text{-}_1$ inducible mediators CCL2, CTGF, and $TGF\text{-}\beta_1$ were examined following experimental lung injury in PAR_1-deficient mice. Thus they will be now each be described in more depth.

1.4.1.6. CCL2 (Monocyte Chemoattractant Protein-1)

CCL2, also previously known as monocyte chemoattractant protein-1, is a member of the CC chemokine family. Chemokines are small secreted proteins of around 60-90 amino acids in length. They all share a similar structure, and are classified into families depending on the patterns of their N-terminal cysteine residues. CC chemokines have 2 adjacent cysteine residues near their N-terminus, and CXC chemokines have a single amino acid between 2 cysteine residues. CX_3C chemokines have 3 amino acids between 2 cysteine residues, and C chemokines have a single cysteine residue at their N-terminus. The major chemokine families are CC and CXC chemokines, and members of these families tend to be chemotactic for mononuclear cells and neutrophils, respectively. In humans CCL2 is localised within a cluster of CC chemokines on chromosome 17, and the murine ortholog of CCL2, which is also known as JE, is localised within a cluster on chromosome 11. Notably a second murine homolog of CCL2, known as MCP-5/CCL12, has been described. This is also situated within the cluster on chromosome 11, and actually is more similar than mouse CCL2 in terms of structure to human CCL2 (Sarafi et al. 1997).

CCL2 is widely expressed on a host of cell types, and expression has been shown to increase in response to mediators associated with injury and inflammation, including IL-1, TNF-α (Sica et al. 1990), IL-6 (Biswas et al. 1998), LPS (Colotta et al. 1992), thrombin (Colotta et al. 1994),(Bachli et al. 2003), factor Xa (Bachli et al. 2003), and also viral infection (Bussfeld et al. 2000). Expression appears to be dependent on the transcription factors AP-1 and NF-κB, and binding sites exist for these exist within the CCL2 promoter.

Chemokines promote cellular effects by binding and activating their seven transmembrane G-protein coupled receptors. Signalling pathways are initiated by G-protein α and βγ subunits, and involve activation of adenylylcyclase, phospholipase C, tyrosine kinases, and ion channels (reviewed in (Keane & Strieter 2000)). The cellular receptor for CCL2 (and also CCL12) is CCR2. CCL2 is chemotactic for monocytes,

but also NK cells (Allavena et al. 1994), T lymphocytes (Carr et al. 1994), basophils (Bischoff et al. 1993), and eosinophils (Dunzendorfer et al. 2001). The mechanism of chemotaxis involves immobilization of CCL2 on gylcosaminoglycans on the surfaces of endothelial and other cells (reviewed in (Moser & Loetscher 2001)). Presentation of CCL2 to CCR2-expressing cells results in integrin activation and arrest, and subsequent transendothelial migration. Cellular migration is mediated by cytoskeletal reorganization, the development of cell polarization, and movement along a gradient of increasing CCL2 concentration on surfaces of extravascular cells and extracellular matrix.

In addition to mediating chemotaxis of inflammatory cells, CCL2 influences the balance of Th1 to Th2-type cytokines in favour of a Th2 profile. CCL2 enhances production of the Th2 cytokine IL-4 by antigen-stimulated OVA-specific TCR transgenic T cells (Karpus et al. 1997). In addition, CCL2-deficient mice produce low levels of the Th2 cytokines IL-4, IL-5 and IL-10 constitutively, and are unable to mount the Th2 immune response following OVA sensitization and challenge that is characteristically observed in wild-type mice (Gu et al. 2000). Interestingly, CCL2 may be protective in a mouse model of endotoxaemia. In this model, mortality is reduced by administration of recombinant CCL2, but increased by administration of anti-CCL2 antibodies. The basis of this effect has been suggested to relate to the CCL2-induced increase in Th2 cytokines such as IL-10 and decrease in Th1 cytokines such as IL-12 and TNF-α (Zisman et al. 1997). However, despite the observed protection in this model, there is still compelling evidence for a pathological role for CCL2 as a pro-inflammatory mediator. CCL2 tissue levels correlate with interstitial cellular infiltration following injury to the lung (Smith 1996), kidney (Prodjosudjadi et al. 1996), large bowel (Banks et al. 2003), brain (Mahad & Ransohoff 2003), and liver (Copple et al. 2003).

In addition to its ability to promote inflammation, CCL2 also promotes fibrosis. Both anti-CCL2 gene therapy and CCR2 deficiency attenuate experimentally-induced lung fibrosis in mice (Inoshima et al. 2004),(Moore et al. 2001),(Gharaee-Kermani et al. 2003). The basis of the profibrotic nature of CCL2 may be its ability to induce TGF-β_1 and procollagen gene expression in fibroblasts (Gharaee-Kermani et al. 1996), to downregulate epithelial cell production of the anti-fibrotic prostanoid PGE_2 (Moore et al. 2003), or to recruit collagen-synthesising

fibrocytes (Moore et al. 2005). Observations of a direct correlation of BALF CCL2 levels with severity of lung injury in ARDS (Goodman et al. 1996), and of serum CCL2 levels serving as a useful biomarker of disease progression in IPF (Suga et al. 1999) support the idea that CCL2 may be involved in the development of human fibroproliferative lung diseases.

1.4.1.7. Connective tissue growth factor (CTGF)

CTGF is a 38kDa polypeptide growth factor, which is encoded by a gene on chromosome 6 in humans, and chromosome 10 in mice. The protein consists of four conserved domains: an insulin-like growth factor (IGF)-binding protein domain, a von Willebrand factor type C domain, a thrombospondin type I repeat domain, and a heparin-binding domain at the C-terminus (**figure 1.5**).

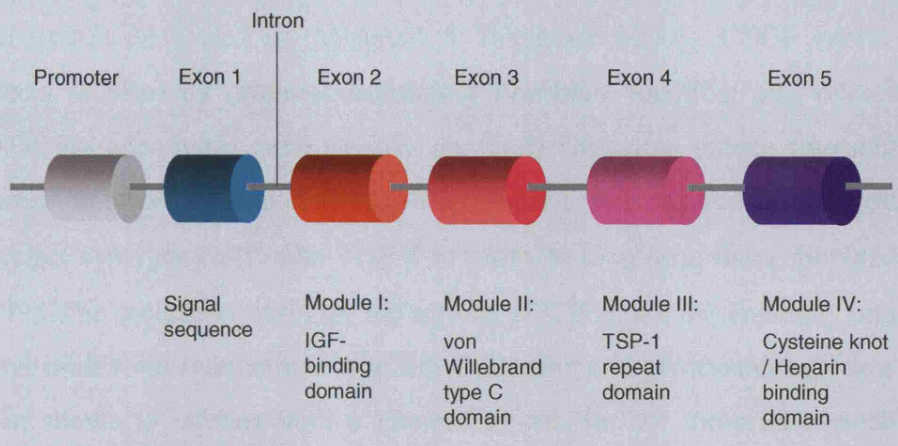


Figure 1.5. Gene organisation of CTGF. The CTGF gene consists of 5 exons. Exon 1 encodes a specific amino acid sequence which allows CTGF to be exported from the endoplasmic reticulum to the Golgi apparatus. Exons 2 to 5 encode 4 conserved modular domans, which have putative functions in IGF binding, oligomerisation, cell attachment, and dimerisation respectively.

CTGF was originally discovered as a secreted product of cultured human endothelial cells (Bradham et al. 1991), although it has since been shown to be expressed by other cell types such as fibroblasts and myocytes; and may, in the context of tissue injury, be additionally expressed in inflammatory cells (Allen et al.

1999), and epithelial cells (Pan et al. 2001). CTGF is an immediate early gene induced in response to TGFβ (Igarashi et al. 1993), but is also induced in response to glucose and insulin (Paradis et al. 2001), mechanical stretch (Schild & Trueb 2002), hypoxia (Kondo et al. 2002), serum (Wenger et al. 1999), angiotensin II (Finckenberg et al. 2003), and thrombin acting via activation of PAR_1 (Chambers et al. 2000). Conversely, CTGF expression in fibroblasts in response to TGFβ can be inhibited by TNF-α (Abraham et al. 2000) and PGE_2 (Ricupero et al. 1999). Expression is likely to require NF-κB transcriptional activity since there are NF-κB binding sites within the CTGF promoter. CTGF plays an important physiological role in processes such as embryogenesis, angiogenesis, tissue differentiation, and wound healing, but is presumed to be pathological in organ fibrosis (reviewed in (Leask & Abraham 2003)). Although the evidence supporting the role for CTGF in organ fibrosis is currently not as strong as for other growth factors such as TGF-β_1, CTGF is highly expressed in the bleomycin model of lung injury (Lasky et al. 1998), as well as in patients with fibrotic lung disease (Allen et al. 1999),(Pan et al. 2001), and fibrosis of other major organs and tissues (reviewed in (Moussad & Brigstock 2000)). CTGF exerts pro-fibrotic effects *in vitro* by directly stimulating fibroblast function; and overexpression of CTGF by adenoviral gene transfer has been shown to induce transient pulmonary fibrosis in mice (Bonniaud et al. 2003). There is also evidence to suggest that CTGF may act synergistically with TGF-β to promote long-term tissue fibrosis (Mori et al. 1999). The molecular basis for the actions of CTGF are incompletely understood, but may result from interactions with other proteins and glycosaminoglycans. CTGF has been shown to interact with a number of cell surface molecules capable of signal transduction, such as IGF-I and IGF-II (Kim et al. 1997), the integrin αvβ3 (Babic et al. 1999), and lipoprotein receptor-associated protein (LRP) (Gao & Brigstock 2003). CTGF can also be internalised from the cell surface in endosomes, and directed to a juxtanuclear organelle, prior to being translocated to the cytosol. Here its phosphorylation by protein kinase C may allow it to play a specific role in the cytosol. In addition, such phosphorylation may allow translocation of CTGF to the nucleus to directly influence gene transcription (Wahab et al. 2001). More recently CTGF has also been shown to be capable of rapidly activating a number of intracellular signalling molecules in human mesangial cells (Wahab et al. 2005).

1.4.1.8. Transforming growth factor-beta-1 (TGF-β_1)

Transforming growth factor beta exists as three isoforms in humans (TGF-β_1,-β_2, and $-\beta_3$). It is able to exert powerful influences over a number of biological processes, such as proliferation, differentiation, migration, and apoptosis. Each isoform is the product of a separate gene, and TGF-β_1 is located on chromosome 19 in humans and on chromosome 7 in mice. All isoforms are initially synthesized as large precursor proproteins, which are later processed intracellularly to yield the mature proteins. This processing gives rise to two products, which subsequently assemble into two distinct dimers. The 65-75kDa dimer from the N-terminal region is known as the latency-associated peptide (LAP), whilst the 25kDa dimer from the C-terminal region is called mature TGF-β. The LAP enables transit of TGF-β away from the cell and renders it biologically inactive; and the complex of LAP with mature TGF-β is known as latent-TGF-β (L-TGF-β). L-TGF-β localizes to the extracellular matrix via binding to latency binding protein (LTBP). Of the three TGF-β isoforms, TGF-β_1 has been most widely studied in the context of experimental fibrosis and fibrotic disease. It is widely accepted as one of the key pro-fibrotic mediators in the bleomycin model (Khalil et al. 1989), as well as in patients with fibrotic lung disease (Khalil et al. 1991),(Khalil et al. 1996). TGF-β_1 is one of the most potent inducers of fibroblast procollagen production examined to date and promotes the differentiation of fibroblasts into highly activated myofibroblasts (Zhang et al. 1996), the predominant fibroblast phenotype present in active fibrotic lesions. TGF-β_1 is also able to induce expression of other pro-fibrotic factors in fibroblasts, most notably CTGF (Igarashi et al. 1993).

Activity of TGF-β may be controlled on a number of levels, namely transcription, stability of mRNA, translation, storage of latent TGFβ, and activation of latent TGFβ. Agents reported to increase transcription of TGF-β_1 include IL-1b (Yue et al. 1994), CCL2 (Gharaee-Kermani et al. 1996), TGF-β_1 itself, PDGF (Villiger & Lotz 1992), bFGF (Qian et al. 1996), angiotensin II (Campbell & Katwa 1997), and IL-13 in combination with TNF-α (Fichtner-Feigl et al. 2006). Activation involves removal of LAP from L-TGFβ, and can occur physiologically following interaction with plasmin, thrombospondin-1, reactive oxygen species, and the αvβ6 and αvβ8 integrins expressed on epithelial cells (reviewed in (Khalil 1999), & (Sheppard 2004)).

Activation can also be induced experimentally *in vitro* by extremes of temperature or pH, chaotropic agents, or substances such as SDS and urea.

TGF-β signalling (reviewed in (Bartram & Speer 2004)) is complex, and involves a series of signalling intermediates known as Smad proteins (**Figure 1.6**). Following association of active mature TGF-β with cell surface TGF-β type II receptors (TβRII), adjacent TGF-β type I receptors (TβRI) are recruited into a heterotrimeric complex. This in turn leads to phosphorylation of TβRI, and subsequent recruitment and phosphorylation of Smad2 and/or Smad3. This phosphorylation enables these Smads to dissociate from the receptor, and form a complex with Smad4, which then translocates to the nucleus to influence gene transcription. This process can be inhibited by Smad6 and/or Smad7. This classical TGF-β_1 Smad signalling pathway also interacts with non-Smad signalling pathways at a number of levels. Such pathways may be initiated by TGF-β_1 itself, or indeed by a different ligand-receptor complex. The Smad signalling pathway may be modulated by phosphorylation of Smads by MAP Kinases, and equally Smads may phosphorylate non-Smad proteins of separate pathways thus modulating their activity.

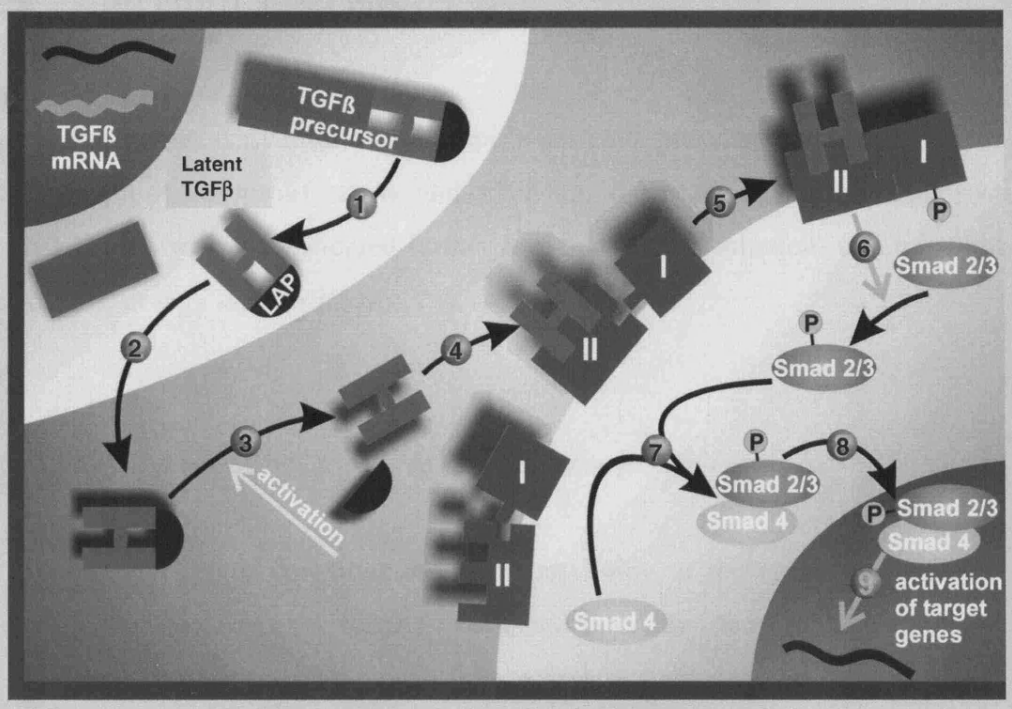


Figure 1.6. The pathway of TGF-β activation and signaling. From TGF-β messenger RNA (mRNA), each TGF-β isoform is initially synthesized as a larger precursor molecule. This is proteolytically cleaved (*1*) and secreted as an inactive protein (*2*) containing a latency-associated peptide (LAP). This is sequestered in the extracellular matrix by binding to latency binding protein (LTBP) *(not shown).* Following activation with release of the LAP (*3*), TGF-β binds to the type II receptor (*4*). This leads to recruitment of the type I receptor (*5*). The consecutively active type II receptor kinase phosphorylates the type I receptor, which propagates the signal downstream through phosphorylation of particular Smads, *ie*, Smad2 and Smad3 (*6*). Smad2 and Smad3 then form a complex with Smad4 (*7*). The complex translocates to the nucleus, where it activates target genes (*8*). P = phosphate molecule.

(Figure taken from Bartram U, 2004)

1.5. Lung epithelial cells

Injury and activation of lung epithelial cells plays an important role in the development of fibroproliferative- and other lung disorders. In the following section, epithelial cells will be considered further in terms of their functions and roles in both normal physiology and in disease.

1.5.1. Function of epithelial cells in normal lung physiology

Epithelial cells constitute a continuous lining of the entire respiratory tract, including the nasal passages, large airways, and extending down to the alveoli situated deep within the lung parenchyma. Besides acting as a physical barrier, epithelial cells perform a number of critical roles that are essential for maintaining normal lung function. In the airways, epithelial cells promote removal of particulate matter from the lungs by secretion of mucus and co-ordinated beating of cilia to propel it, and any entrapped particles, upwards to the central airways from where it is coughed out, and then either expectorated or swallowed. Airway epithelial cells also produce a number of mediators which regulate tone of underlying smooth muscle tone. In the distal respiratory tract, alveolar epithelial cells are closely opposed to capillary endothelial cells, and are fundamental in enabling gaseous exchange between the alveolar cavity and the blood. Alveolar epithelial cells also secrete surfactant, which prevents airspace collapse by lowering surface tension on the alveolar surface. In addition, certain lung epithelial cells throughout the respiratory tract promote innate immune responses by release of pro-inflammatory mediators in response to specific stimuli, and thus recruit inflammatory cells to sites of epithelial injury. Bioactive mediators produced constitutively, and in response to injury by epithelial cells have profound effects on the function of resident inflammatory cells, neighbouring epithelial cells and underlying mesenchymal cells.

1.5.1.1. Airway epithelial cells

Airway epithelial cells are pseudostratified in the proximal bronchi but form a less complex monolayer in more distal parts of the bronchial tree. The majority of epithelial cells lining the airway are ciliated columnar cells, but additionally present are basal cells, goblet cells, Clara cells, and neuroendocrine cells. Basal cells are small epithelial cells which contact the basement membrane but not the airway lumen. They are a stem cell population from which other airway epithelial cells develop. They are the second most common cell type in proximal airways, but their relative proportion is far less distally. Goblet cells and Clara cells are primarily secretory cells. Goblet cells predominate in larger airways, and secrete mucus, although mucus is also produced by abundant tracheobronchial glands extending into the submucosa and emptying into the airway lumen. Clara cells are the predominant cell type of the bronchiolar epithelium. They form a simple columnar and non-ciliated epithelium, but exhibit some functional similarity with more proximal airway epithelial cells, including expression of Clara Cell Specific Protein (CCSP)(Broers et al. 1992), (Cowan et al. 2000),(Yao et al. 1998) and Surfactant Protein A (Broers et al. 1992). Clara cells function as stem cells of the distal airways with the ability to differentiate into more Clara cells, ciliated cells, or Goblet cells. Neuroendocrine cells are very sparsely distributed along the basement membrane in distal airways and secrete hormones and active peptides. They are thought to play an important role in lung development.

In addition to native epithelial cells, a number of other cell types migrate to and subsequently reside within or adjacent to lung epithelial cell layers. Abundant sensory nerves from subepithelial plexuses innervate airway epithelial cells, particularly neuroendocrine cells. Immune cells, such as macrophages, lymphocytes, dendritic cells, and mast cells also reside within epithelial cell layers. By interaction with these, the lung epithelium is able to influence adaptive as well as innate immune responses.

1.5.1.2. Alveolar epithelial cells

Repeated branching of the bronchial tree terminates in respiratory bronchioles, which subsequently lead to alveolar ducts, and finally to alveolar sacs. Around 99% of the internal surface area of the lung is covered by alveolar epithelial cells (reviewed in (Crandall & Matthay 2001)). Two morphologically distinct types of alveolar epithelial

cells exist: Type I cells comprise about 40% of the alveolar epithelial cell population but cover 90% of its surface area (Stevens & Lowe 1992). They are large flattened squamous cells joined to each other by tight junctions, and to neighbouring type II cells by gap junctions. They are highly adapted for their principal function of gas exchange; although they also have other functions including regulation of alveolar water and ion transport (Johnson et al. 2002), processing of peptides within the alveolar lumen (Nagae et al. 1993), and modulation of alveolar macrophage function via the expression of ICAM-1 (O'Brien et al. 1999). Because of their large surface area and complex cytoplasmic extensions, each cell may form part of the epithelial surface of more than one alveolus. Type II alveolar epithelial cells comprise 60% of the alveolar epithelial cell population but cover only 10% of the alveolar surface area. They are cuboidal cells typically situated in the corners of alveoli. They have microvilli on their luminal surface, and are highly metabolically active. Their cytoplasm contains abundant organelles, including mitochondria, endoplasmic reticulum, and lamellar bodies. The latter represent storage granules of synthesised surfactant. Type II cells have multiple functions. They have important progenitor function, and are effectively the stem cell of the alveolus. Type II cells are able to proliferate to produce additional type II cells, but can also differentiate into type I cells. They synthesise and release surfactant: a mixture of phospholipids and specific proteins, which forms a layer over the wall of the alveolar sac, and prevents alveolar collapse by reducing surface tension. Type II cells play an important role in controlling alveolar fluid balance via regulation of apical membrane sodium channels (Eaton et al. 2004). They can influence the immune response by releasing agents such as RANTES, CCL2 (O'Brien et al. 1998), and GM-CSF (Christensen et al. 1995) to attract and/ or stimulate alveolar macrophages and other inflammatory cells. Type II cell-derived surfactant protein-A (SP-A) may also modulate certain aspects of function of alveolar macrophages including their release of reactive oxygen species (Weissbach et al. 1994).

1.5.2. Epithelial injury within the lung

Epithelial injury is common to a number of important lung disease entities. The outcome is governed by the nature and site of this injury. For instance, in asthma, chronic inflammation initiated by allergen exposure, and associated with eosinophils

and IgE-dependent mast cell-derived mediators, results in widespread epithelial injury to bronchi and bronchioles, but not alveoli. This promotes airway mucosal inflammation and oedema, increased mucus production, and bronchial smooth muscle contraction. In COPD an inflammatory response distinct from that in asthma, and involving a predominance of macrophages, cytotoxic T cells, and neutrophils affects predominantly small airways and lung parenchyma. This is associated with bronchiolar and alveolar epithelial injury, and results in small airway fibrosis and alveolar destruction and emphysema.

ARDS and IPF are associated with a predominantly initial alveolar epithelial injury. Since around 99% of the internal surface area of the lung is covered by alveolar epithelial cells, the alveolar epithelium is exposed to a multitude of potentially injurious insults, and is thus susceptible to injury. In ARDS, these injuries affect both endothelial and epithelial cells, leading to loss of endothelial/epithelial barrier integrity, alveolar flooding, and inflammation. In IPF multiple idiopathic injuries are thought to occur sequentially throughout large areas of the lungs. In both instances, a subsequent abnormal repair response involving abnormal epithelial-mesenchyme interactions may promote alveolar fibrosis.

1.5.3. The bleomycin model and epithelial injury

In the experimental work outlined in this thesis, pulmonary fibrosis was induced in C57/BL/6 mice by the administration of intra-tracheal bleomycin. The multiple biological mechanisms involved in the development of human lung fibrosis are of a complexity that is impossible to recreate in an *in vitro* system. An animal model is able to provide a realistic representation of many of these mechanisms, and is fundamental to any study that aims to further understanding of disease processes. C57/BL/6 mice were used because they are known to be particularly sensitive to bleomycin-induced lung fibrosis (Kolb et al. 2002),(Brody et al. 2002). Susceptibility may relate to production of a greater proportion of the Th2 cytokines IL-4 and IL-13 in response to injury, since following bleomycin these cytokines are upregulated in bleomycin-sensitive C57/BL6 mice but not bleomycin-resistant BALBc mice (Cavarra et al. 2004).

Following intra-tracheal administration of bleomycin, the early response is characterized by a dramatic increase in microvascular leak and inflammatory cell

recruitment (Thrall & Scalise 1995). Inflammation in the bleomycin model is associated with a increasing levels of number of cytokines, including the Th_1 cytokine tumour necrosis factor-alpha (TNF-α) (Piguet et al. 1989),(Ortiz et al. 1998),(Gurujeyalakshmi et al. 2000),(Cavarra et al. 2004), although in subsequent progression to fibrosis, Th_2 cytokines such as IL-4 and IL-13 (Cavarra et al. 2004) are likely to be important. The inflammatory response is succeeded by intra-alveolar fibrosis, focally dense fibrosis, and subpleural fibrosis which are most prominent at 14 and 21 days after instillation (Izbicki et al. 2002). It should be noted that murine bleomycin-induced lung injury represents a model of lung injury and fibrosis; it does not represent a model of any specific human disease. However key features of the bleomycin model such as widespread epithelial injury and activation, fibroblast proliferation and collagen synthesis, and production of pro-fibrotic mediators by resident lung cells, are central to the bleomycin model and to the pathophysiology of fibroproliferative lung disorders such as ARDS and IPF. Intra-tracheal bleomycin induces an acute inflammatory lung injury progressing to fibrosis that may more closely reflect the pathophysiology of ARDS than that of IPF. However, microarray studies have confirmed that gene expression profiles of human IPF lung have many similarities to those of bleomycin-injured mouse lung (Kaminski 2003).

Bleomycin is a chemotherapeutic agent which exhibits dose-dependent pulmonary toxicity. It exerts its toxic effect by binding to guanine-cytosine-rich portions of DNA, together with divalent metal ions including iron and copper. The bound iron is able to produce highly reactive free radicals from molecular oxygen, which in turn can cleave DNA (Dorr 1992). The lung is particularly susceptible to bleomycin-induced injury since it lacks bleomycin hydrolase, a naturally occurring cysteine proteinase found in other organs, which degrades it (Bromme et al. 1996). The pulmonary response to bleomycin depends on the route of administration. Intra-tracheal bleomycin immediately affects the alveolar and bronchiolar epithelium, and ultimately leads to peri-bronchiolar fibrosis; whereas intravenous or intraperitoneal administration initially affects pulmonary endothelial cells, and ultimately leads to perivascular and subpleural fibrosis (Harrison, Jr. & Lazo 1987).

Common to all routes of administration of bleomycin is early widespread epithelial damage. Necrosis of type I alveolar epithelial cells ensues immediately following bleomycin injury. Their vulnerability is probably largely due to their exposed attenuated cytoplasm. Type II alveolar epithelial cells are also lost following

bleomycin injury, although this may be via apoptosis rather than necrosis. Excessive epithelial apoptosis following bleomycin has been proposed to be a mechanism promoting fibrogenesis by releasing neighbouring lung fibroblasts from an inhibitory influence of lung epithelial cells (Hagimoto et al. 1997). Epithelial apoptosis is promoted by a number of mechanisms including exposure to reactive oxygen species (Wallach-Dayan et al. 2005), TNF-α (Wang et al. 2000a), and Fas ligand (Hagimoto et al. 1997). The mechanism appears to involve binding of angiotensin peptides to the AT1 receptor on the epithelial cells. These angiotensin peptides may be produced in an autocrine fashion by epithelial cells themselves in response to the aforementioned mediators (Li et al. 2003), or by neighbouring fibroblasts (Uhal et al. 1995).

The cellular source of reactive oxygen species following bleomycin are type II alveolar epithelial cells and macrophages (Inghilleri et al. 2005). The importance of reactive oxygen species in bleomycin-induced damage is supported by the observation that administration of the antioxidant N-acetyl cysteine attenuates the extent of bleomycin-induced lung injury (Hagiwara et al. 2000). Epithelial cells influence the oxidant/ antioxidant balance following bleomycin by regulation of intracellular levels of antioxidants. For instance, levels of glutathione are decreased in type II alveolar epithelial cells following bleomycin injury (Karam et al. 1998). Conversely, levels of the antioxidant thioredoxin are increased in airway epithelial cells following bleomycin, and may afford protection from injury in these cell types (Gon et al. 2001).

An important function of type II alveolar epithelial cells is the production and secretion of surfactant, comprising phospholipids and specific surfactant proteins, to prevent alveolar collapse. Lung levels of phospholipids and surfactant apoproteins B and C are reduced in the bleomycin-injured lung (Osanai et al. 1991),(Savani et al. 2001), and, their deficiency may predispose to alveolar atelectasis, leading to hypoxaemia and respiratory insufficiency. Studies involving surfactant protein C-knockout ($SFTPC^{-/-}$) mice have additionally suggested that surfactant protein C may additionally limit lung inflammation, inhibit collagen accumulation, and promote resolution of normal lung following bleomycin injury (Lawson et al. 2005).

Epithelial and endothelial cell loss results in inadequate epithelial/ endothelial barrier integrity. Vascular leak, possibly in concert with dyregulation of epithelial gap junction proteins (Abraham et al. 1999) and the water channel aquaporin-5 (Gabazza et al. 2004) produce alveolar flooding with protein rich oedema fluid. Local activation of coagulation is initiated by upregulation of tissue factor on a number of resident

lung cells and extravasation of coagulation proteinase zymogens and fibrinogen from the vasculature. This allows generation of coagulation proteinases such as thrombin, and ultimately the formation of fibrin, which is deposited as hyaline membranes on the denuded basement membrane of the alveolar walls. Such hyaline membranes are classical pathological features of the bleomycin model (Jones & Reeve 1978) and ARDS (Anderson & Thielen 1992). Following bleomycin, but also in fibroproliferative lung disorders, alveolar fibrin persistence is maintained by a procoagulant and antifibrinolytic environment. Fibrin may exert a pro-fibrotic effect *in vivo*, since administration of u-PA, which favours fibrin clearance, attenuates bleomycin-induced fibrosis (Gunther et al. 2003).

Evidence for the ability of epithelial cells to promote a pro-coagulant state comes from i*n situ* hybridisation studies, which show that tissue factor expression is upregulated predominantly on alveolar epithelial cells following bleomycin (Olman et al. 1995). Others have shown that epithelial cells also express PAI-1 and uPA, at least *in vitro* (Gross et al. 1991), which raises the possibility that they may also have the potential to modulate fibrinolysis as well as coagulation.

Following bleomycin, type II alveolar epithelial cells adopt a hyperplastic activated phenotype (Karam et al. 1998),(Kunugi et al. 2001),(Folkesson et al. 1998),(Aso et al. 1976), and these are a characteristic feature of this model; but also of ARDS (Stanley et al. 1992) and of IPF (Iyonaga et al. 1997),(Sakai et al. 1997). Small airways are also affected by bleomycin injury (Daly et al. 1998), (Kawamoto & Fukuda 1990), and in a process known as bronchiolization, bronchiolar epithelial cells proliferate and colonize alveolar spaces; and some of the cuboidal epithelial cells in the region of alveolar injury after bleomycin are likely to be derived from the distal conducting airways (Kawamoto & Fukuda 1990),(Betsuyaku et al. 2000). The role of airway epithelial cells in the injury/ healing response to bleomycin is further supported by the observation of changes in gene expression following bleomycin in these cell types (Betsuyaku et al. 2001, Betsuyaku et al. 2003).

The inflammatory response following bleomycin involves polymorphonuclear cells, lymphocytes and monocytes/ macrophages. Clearly the initiation and propagation of this response involves a number of distinct mediators and processes, and is dependent on a range of resident lung cell types, although it seems likely that epithelial cells play an important part in this regard. Bleomycin stimulates the release of mediators which promote inflammatory cell recruitment *in vitro*, in particular, the

neutrophil chemoattractants: LTB4, IL-8 and G-CSF, and the mononuclear cell chemoattractant CCL2 (Sato et al. 1999). It is likely that oxidative stress is initially involved in the expression and release of pro-inflammatory mediators (reviewed in (Crapo 2003)), but additional mechanisms may include activation of toll-like receptor 2. Both alveolar (Armstrong et al. 2004) and small airway epithelial cells (Ritter et al. 2005) express abundant toll-like receptor 2 (TLR2), and bleomycin has been recently shown to stimulate cytokine secretion via TLR2 in monocytes (Razonable et al. 2005).

Epithelial cells are also a potential source of a number of potent pro-fibrotic mediators which have been implicated as promoting the development of injury and fibrosis in the bleomycin model. TNF-α is produced by type II alveolar epithelial cells following lung injury (Song et al. 1998), and administration of antibodies directed against TNF-α attenuate alveolar damage and fibrosis in response to bleomycin (Piguet et al. 1989). The CC chemokines MIP-1α and CCL2; and the CXC chemokine MIP-2 (the murine homolog of IL-8) are expressed by injured alveolar and/or bronchial epithelial cells (Smith et al. 1994),(Lundien et al. 2002),(Witherden et al. 2004),(Vanderbilt et al. 2003). Antibodies directed against MIP-1α (Smith et al. 1994) and MIP-2 (Keane et al. 1999b), and anti-CCL2 gene therapy (Inoshima et al. 2004) all attenuate the fibrotic response after bleomycin. Lung epithelial cells also produce TGF-β_1 (Azuma et al. 2005a) and PDGF (Song et al. 1998) following bleomycin injury, and similarly, the extent of fibrosis is attenuated by administration of TGF-β_1 blocking antibodies (Giri et al. 1993), and by administration of the PDGF receptor tyrosine kinase inhibitor: imatinib mesylate (Daniels et al. 2004).

Expression of the αvβ6 integrin is increased on injured airway (Pilewski et al. 1997) and injured alveolar epithelium, but is absent from both these cell types in the normal lung (Breuss et al. 1995). A major mechanism for TGF-β_1 activation *in vivo* is likely to occur via this integrin, since αvβ6-integrin$^{-/-}$ mice are protected from bleomycin-induced lung fibrosis (Munger et al. 1999). Hyperplastic proliferating epithelial cells are evidently an important source of TGF-β_1 production following bleomycin, and yet they may specifically adapt to be resistant to its antiproliferative effects by downregulation of the TGF-β Receptor-1 that is necessary for TGF-β_1 signal transduction (Khalil et al. 2002).

Finally, other mediators characteristically associated with inflammation and/ or fibrosis following bleomycin, including IL-1, IL-6, IL-4, IL-13, amd CTGF are all

produced by injured or proliferating lung epithelial cells (Cavarra et al. 2004),(Howell et al. 2001),(Mattoli et al. 1991),(Bartalesi et al. 2005),(Pan et al. 2001), implicating epithelial cells as a potential source of these mediators.

Following epithelial injury, hyperplastic epithelial cells proliferate and spread along the alveolar septa and differentiate into type I cells in order to repair the alveolar epithelial defect. Type II alveolar epithelial cells produce a number of growth factors which can act in an autocrine fashion to stimulate epithelial repair. These include hepatoma-derived growth factor (HDGF) (Mori et al. 2004), hepatocyte growth factor (HGF) (Douglas et al. 2002), and Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) (Sato et al. 2000). Diminished production of the latter following bleomycin may be a mechanism promoting fibrogenesis (Christensen et al. 2000). In addition, in the context of fibrogenesis, differentiation of type II into type I cells may be impaired, leading to increased numbers of dysfunctional type II cells and transitional epithelial cell types (Kasper & Haroske 1996). Thus epithelial cells are potentially key players in both initiating and perpetuating injury, but also in promoting subsequent resolution and repair following bleomycin. Their influence on injury/repair is likely to depend on the nature of their interactions with neighbouring resident lung cells such as myofibroblasts, and the local milieu of bioactive mediators present.

1.5.4. Epithelial injury in fibroproliferative lung disorders

As mentioned previously, the bleomycin model recapitulates key pathophysiological and biochemical features of human fibroproliferative lung disorders and has enabled major progress in our understanding of them. Widespread epithelial injury and activation is pivotal to both ARDS and IPF, although the pathogenesis of these disorders differs to some extent.

As mentioned earlier, the pathogenesis of ARDS involves exudative, proliferative, and fibrotic phases that follow widespread epithelial and/or endothelial injury. Key pathogenetic events are illustrated in **figure 1.7**.

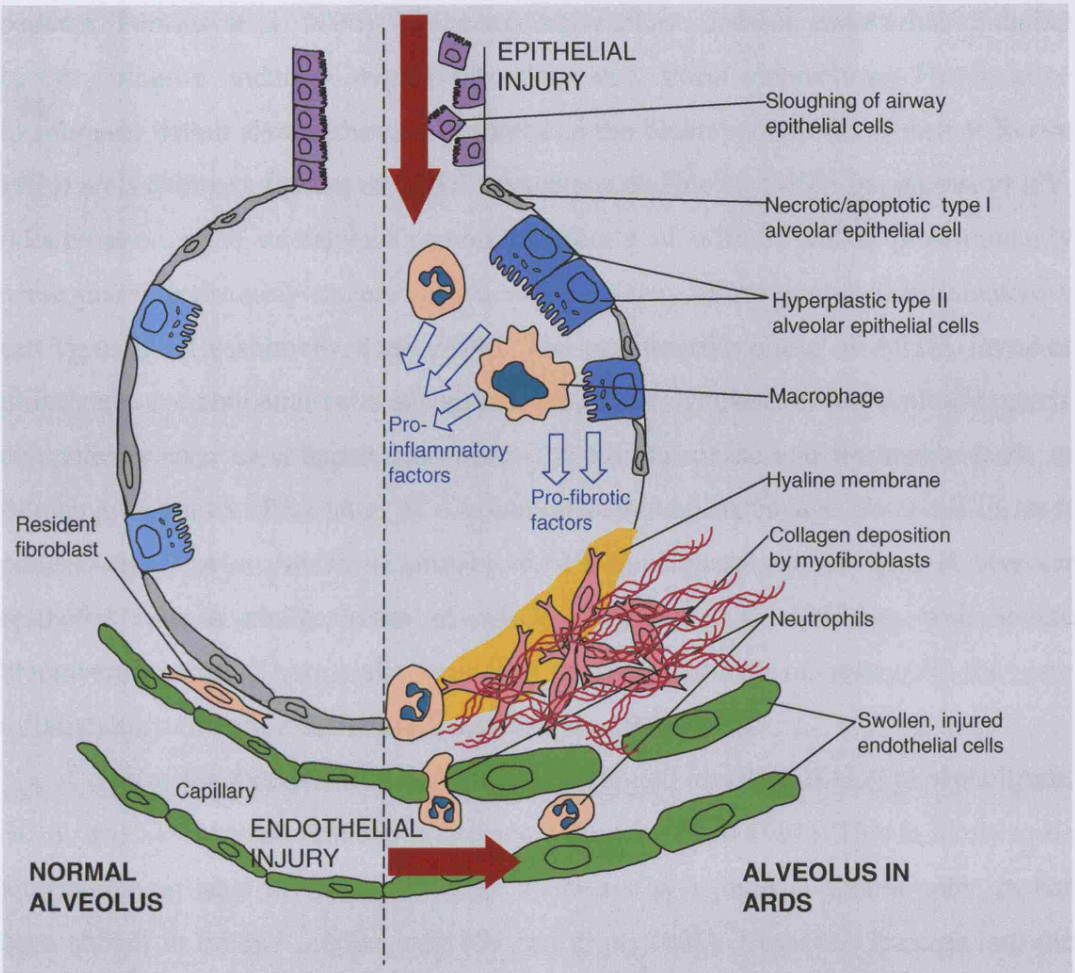


Figure 1.7. Hypothetical scheme of pathogenetic events in ARDS leading to fibrosing alveolitis

Epithelial injury leads to loss of vulnerable type I alveolar epithelial cells, and possibly airway epithelial cells. Co-existent endothelial injury leads to swollen activated endothelial cells which promote recruitment of inflammatory cells by upregulation of adhesion molecule and chemokine expression. Activated endothelial cells contract causing vascular leak and facilitating trafficking of leukocytes through the interstitium into the alveolar airspace, which is flooded with protein-rich oedema fluid. Local upregulation of tissue factor together with vascular leak permits generation of thrombin and fibrin. Extensive protein-rich fibrin-containing hyaline membranes form on the denuded basement membrane of the alveolar wall. Activated neutrophils, macrophages, and epithelial cells release pro-inflammatory and pro-fibrotic factors which perpetuate local cellular injury and inflammation, but also promote recruitment, proliferation, and activation of resident fibroblasts. Myofibroblasts synthesize and deposit extracellular matrix proteins such as collagen within interalveolar septae and in areas of fibrinous matrix (hyaline membranes) within the alveolus.

The epithelial injury in ARDS may additionally involve airway as well as alveolar epithelial cells, since bronchial epithelial cells are present in BALF of these

patients (Perkins et al. 2006). In the exudative phase, loss of endothelial/epithelial barrier integrity induces alveolar flooding and fibrin deposition. The hyaline membranes within alveoli that are observed in the bleomycin model (Jones & Reeve 1978) are a hallmark feature of ARDS (Anderson & Thielen 1992). In contrast to IPF, inflammation is an undisputed prominent feature of ARDS, and is predominantly neutrophilic in the early stages, but macrophages may be the dominant inflammatory cell type later (Hashimoto et al. 2000). The proliferative phase of ARDS involves proliferation of epithelial cells and fibroblasts. Their synthesis of extracellular matrix components such as collagen constitutes the fibrotic phase and ultimately leads to fibrosing alveolitis. This latter is a common pathological change observed in most patients dying with ARDS (Lamy et al. 1976). Hyperplasia of type II alveolar epithelial cells is characteristic of ARDS (Stanley et al. 1992) as well as the bleomycin model. These cells represent a likely source of many of the pro-inflammatory and pro-fibrotic mediators found in BALF.

Surfactant function is abnormal in ARDS, and levels of BALF phospholipids, SP-A, and SP-B are all reduced in patients (Gregory et al. 1991). This is likely to be attributable, at least in part, to reduced synthesis by injured epithelial cells, as has been shown in injured murine lung (Savani et al. 2001). However, leakage into the circulation is probably also a contributing factor, and increased levels of surfactant proteins B and C are measurable in serum of ARDS patients, and may serve as biomarkers of physiological severity (Doyle et al. 1997). Surfactant deficiency in ARDS leads to end-expiratory alveolar collapse, atelectasis, an increase in right-to-left shunt and hypoxaemia. Surfactant replacement improves lung physiology and mortality in severe neonatal respiratory distress syndrome, but has thus far not proved efficacious in adults.

Lungs in patients with ARDS are difficult to ventilate because of increased compliance as a result of alveolar flooding, fibrin deposition, and atelectatic alveoli. Consequently high pressures (particularly positive end expiratory pressure: PEEP) are needed to keep alveoli open and facilitate gas exchange. However, these may cause ventilator-induced lung injury. In animal models, this has been shown to be associated with production and release of a number of pro-inflammatory cytokines (von Bethmann et al. 1998) including MIP-2, the murine homolog of IL-8 (Held et al. 2001). Likely sources of these mediators are hyperplastic type II alveolar epithelial cells, and pulmonary microvascular endothelial cells (reviewed in (Pinhu et al. 2003)).

Mechanical stretch induces expression and release of IL-8 from A549 alveolar epithelial cells (Vlahakis et al. 1999) *in vitro*, and levels of this mediator in BALF are raised in experimental ventilator-induced lung injury (Park et al. 2005) in animals, but also correlate with physiological severity and outcome in ARDS patients (Chollet-Martin et al. 1993).

Activated hyperplastic epithelial cells are a pathological feature of IPF (Iyonaga et al. 1997),(Sakai et al. 1997), just as they are of ARDS and of the bleomycin animal model. The pathogenetic mechanisms of IPF are illustrated in **figure 1.8**.

Clusters of activated and proliferating fibroblasts/ myofibroblasts occur in the vicinity of areas of focal epithelial cell destruction and exposed basement membrane. Adjacent hyperplastic epithelial cells represent the likely source of the pro-fibrotic mediators driving synthesis of extracellular matrix proteins by these fibroblastic foci. In support of this, immunohistochemical studies involving IPF biopsies have demonstrated that the major site of localization of TNF-α and TGF-β_1 is type II alveolar epithelial cells (Kapanci et al. 1995). Injured and/ or activated airway epithelial cells may also play a role in the production of pro-fibrotic mediators in IPF, and bronchiolization is apparent in IPF (Chilosi et al. 2003) just as it is in the bleomycin model. Prominent bronchial immunostaining for osteopontin (Kadota et al. 2005) and endothelin-1 (Saleh et al. 1997) is apparent in lungs of IPF patients, supporting airway epithelium as a potential source of pro-fibrotic mediators.

A number of epithelial-derived proteins are detectable in the serum of IPF patients, which most likely relates to increased release from injured or activated epithelial cells, and subsequent leakage into the circulation. Examples of such proteins are Cytokeratin 19 (Nakayama et al. 2003), SP-A and SP-D (Greene et al. 2002), Carcinoembryonic Antigen (Hadjiliadis et al. 2001), HGF (Yamanouchi et al. 1998), and KL-6 (Yokoyama et al. 1998). These proteins have been shown to have potential value as biomarkers of disease severity and/ or monitoring response to treatment.

Finally, TGF-β_1 has also been shown to have the potential to promote transition of alveolar epithelial cells toward a myofibroblast-like phenotype *in vitro* (Willis et al. 2005),(Kasai et al. 2005). Cells co-expressing both epithelial and mesenchymal markers have been identified in lungs of IPF patients (Willis et al. 2005), consistent with these cells being of a transitional phenotype somewhere

between epithelium and mesenchyme. If such epithelial-mesenchymal transition is able to occur *in vivo*, it may represent an important further role for epithelial cells in fibrogenesis in IPF.

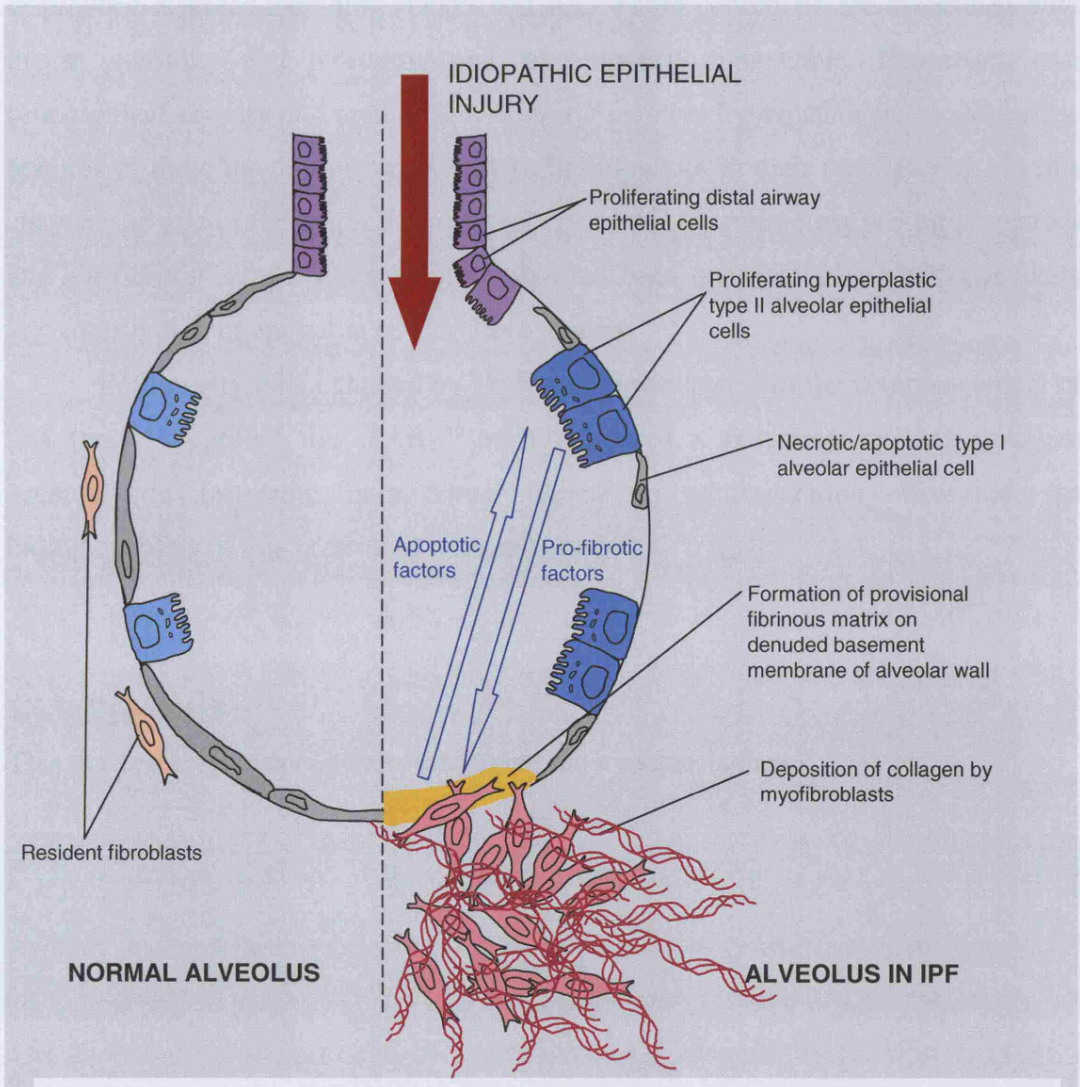


Figure 1.8. Hypothetical scheme of pathogenetic events in IPF

Idiopathic epithelial injury leads to loss of type I alveolar epithelial cells and exposure of underlying basement membrane. Vascular leak and local upregulation of tissue factor expression results in generation of thrombin and fibrin. Type II alveolar epithelial cells proliferate and spread over denuded basement membrane and undergo reactive hyperplasia. Distal bronchiolar epithelial cells may also proliferate and spread into the alveolar space (bronchiolization). Activated epithelial cells release pro-fibrotic factors inducing recruitment, proliferation, and activation of resident fibroblasts. Myofibroblasts may promote epithelial cell apoptosis and disruption of the basement membrane thus preventing normal epithelial repair. Myofibroblasts also sythesize and deposit extracellular matrix proteins such as collagen within interalveolar septae and in areas of fibrinous matrix within the alveolus. Normal matrix degradation/ turnover does not occur because of a local imbalance between TIMP and MMP activity.

1.6. Summary, hypothesis and specific aims of this thesis

Pulmonary fibrosis is a pathological process common to a number of respiratory diseases including ARDS and IPF. These conditions are associated with major morbidity and mortality, and are currently untreatable. Excessive local procoagulant activity and epithelial cell injury/ reactive hyperplasia are characteristic features of these conditions, and likely to be important in their pathogenesis. In this chapter, the role of the major thrombin receptor PAR_1 in promoting pro-inflammatory and pro-fibrotic cellular effects of thrombin has been reviewed, along with the likely key contribution of epithelial cells to fibrogenesis.

Preliminary data obtained by Dr David Howell prior to the commencement of this thesis suggested that $PAR_1^{-/-}$ mice exhibited a reduction in lung collagen accumulation following intratracheal bleomycin administration. However, the biological basis of this protection were not known.

1.6.1. Hypothesis:

This thesis therefore specifically addressed the hypothesis that.

PAR_1 activation promotes lung injury and fibrosis via the release of a number of specific secondary mediators; with the pulmonary epithelium representing an important cellular source of these mediators.

1.6.2. Aims:

The specific aims of this thesis were:

1. To determine the contribution of PAR_1 to lung inflammation and fibrosis in response to lung injury by evaluating these responses in $PAR_1^{-/-}$ and WT mice following bleomycin-induced lung injury

2. To identify the secondary mediators by which PAR_1 drives inflammation and fibrosis following lung injury by comparing the levels of PAR_1 inducible

mediators in lungs of $PAR_1^{-/-}$ and WT mice following bleomycin-induced lung injury.

3. To determine whether PAR_1 activation on lung epithelial cells promotes the release of pro-inflammatory and pro-fibrotic secondary mediators by using cultured lung epithelial cells.

2. MATERIALS & METHODS

2.1. Materials

As mentioned previously $PAR_1^{-/-}$ mice were originally generated by the insertion of a neocassette into the PAR_1 gene to disrupt exon 2 which encodes transmembrane domains 1-7 (Connolly et al. 1996). The resulting DNA construct was electroporated into embryonic stem cells, where it incorporated into chromosomal DNA by homologous recombination. Neomycin-resistant clones were implanted into mouse blastocyst cavities, and resulting chimeras were bred and genotyped. $PAR_1^{-/-}$ offspring of these were then extensively backcrossed for 10 generations onto the C57/BL/6 background (Cunningham et al. 2000). A colony of homozygous $PAR_1^{-/-}$ mice was subsequently established at UCL through collaboration with Prof P Tipping (Monash University, Australia). A colony of C57/BL/6 mice obtained from *Harlan UK (Oxon, UK)* were established at the same time at UCL, and served as wild-type controls.

ELISA kits for mouse IL-6, TNF-α, and IL-10 were from *RnD systems (Oxon, UK)*, and for mouse CCL2 from *BD Pharmingen (San Diego, CA, USA)*. Paired antibodies MAB679 and BAF279 for the human CCL2 ELISA were obtained from *RnD systems*; and human recombinant CCL2 protein standard was from *Peprotech (Rocky Hill, NJ, USA)*.The lactate dehydrogenase (LDH) activity assay kit was from *Sigma-Aldrich (Dorset, UK)*. Antibodies specific for mouse CCL2 (sc-1784), mouse/human CTGF (L-20) and mouse/human TGF-β_1 (sc-146: which recognizes latent and active TGF-β_1) were obtained from *Santa Cruz Biotechnology Inc (San Diego, CA, USA)*. Mouse specific PAR_1 antibodies, raised against the peptide SFFLRNPSENTFELVPL-NH_2 (abbreviated SFFL), in rabbits, and purified by affinity chromatography (Jenkins et al. 1993), were a generous gift from Professor Eleanor Mackie (University of Melbourne, Australia). Recombinant human thrombin and factor Xa were obtained from *Calbiochem (Darmstadt, Germany)*.

A549, BEAS-2B, HFL-1, and MLF cell lines were obtained from the *American Type Culture Collection (ATCC)* via *LGC Promochem (Middlesex, UK)*. The MLE-15 cell line was a generous gift from Professor Jeffrey A. Whitsett (Cincinatti Children's Hospital Medical Center, Cincinatti, Ohio, USA). Primary

human lung alveolar epithelial cells were a generous gift from Professor Terry Tetley, Lung Cell Biology, NHLI, Imperial College, London.

DMEM and F12-K tissue culture media were from *Gibco BRL, Paisley, UK.* DCCM-1 tissue culture medium was from Biological Industries *(Kibbutz Beit Haemek, Israel).* Tissue culture flasks and plates were all obtained from *Nunc (Roskilde, Denmark).* TFLLR-NH_2 and FTLLR-NH_2 peptides were synthesized by Professor Robert Mecham (University of Washington, St Louis, MO, USA). The AYPGKF peptide was kindly provided by Dr James Moffat (Kings College London). The PAR_1 antagonist RWJ-58259 was a gift from Dr Claudia Derian (Johnson & Johnson Pharmaceutical R&D, Philadelphia, USA). The protein kinase C inhibitor Ro-31-8425 and pertussis toxin were obtained from *Calbiochem.* Mink lung epithelial cells stably transfected with the PAI-1-promoter-luciferase reporter gene, for use in the TGF-β_1 bioassay, were kindly provided by Dr Dan Rifkin (Department of Cell Biology, New York University School of Medicine, New York, USA).

Primers used in real-time RT-PCR studies are listed in **table 2.1**. Murine CTGF, TGF-β_1 and 36B4 primers were designed by Dr Bin Shan (Tulane University, New Orleans, USA). All other primers were designed in-house at UCL with the assistance of Dr Chris Scotton (Centre for Respiratory Research, UCL).

Gene	Primers	Product size
Mouse PAR_1	For 5'-AGCCAGCCAGAATCAGAGAG-3' Rev 5'-AGGGGGACCAGTTCAAATGTA-3'	96bp
Mouse CTGF	For 5'-GCAGCGGTGAGTCCTTCC-3' Rev 5'-AATGTGTCTTCCAGTCGGTAGG-3'	232bp
Mouse TGF-β_1	For 5'-GGATACCAACTATTGCTTCAGCTCC-3' Rev 5'-AGGCTCCAAATATAGGGGCAGGGTC-3'	155bp
Mouse 36B4	For 5'-CGACCTGGAAGTCCAACTAC-3' Rev 5'-ATCTGCTGCATCTGCTTG-3'	109bp
Mouse CCL2	For 5'-AGCTCTCTCTTCCTCCACCAC-3' Rev 5'-CGTTAACTGCATCTGGCTGA-3'	111bp
Mouse/Human 18S	For 5'-TTGACGGAAGGGCACCACCAG-3' Rev 5'-GCACCACCACCCACGGAATCG-3'	131bp
Human PAR_1	For 5'-CCATCGTTGTGTTCATCCTG-3' Rev 5'-GACCCAAACTGCCAATCACT-3'	148bp
Human PAR_4	For 5'-GAAGGCTGTACTGGGTCGAA-3' Rev 5'-AAGTGACCTCCGCTAGTGACA-3'	119bp
Human CTGF	For 5'-GCAGGCTAGAGAAGCAGAGC-3' Rev 5'-TGGAGATTTTGGGAGTACGG-3'	108bp
Human TGF-β_1	For 5'-AAGGGCTACCATGCCAACT-3' Rev 5'-CCGGGTTATGCTGGTTGTA-3'	103bp
Human CCL2	For 5'-AGCAAGTGTCCCAAAGAAGC-3' Rev 5'-CATGGAATCCTGAACCCACT-3'	94bp

Table 2.1: Primers used for real-time RT-PCR

2.2. Bleomycin model of lung injury and fibrosis

2.2.1. Surgical procedures, and harvesting of tissue and lavage fluid

Mice were housed in a specific pathogen-free facility, and all procedures were performed on mice aged between 6 and 12 weeks of age in accordance with the Home Office Animals Scientific Procedures Act (HMSO 1986). Bleomycin *(Nippon Kayaku Co. Ltd, Tokyo, Japan)* or saline was administered by a single intra-tracheal injection (1mg/kg body weight in 50µl saline) into a surgically exposed trachea under halothane anaesthesia. Mice were later sacrificed by intraperitoneal injection of pentobarbitone, and severing of the abdominal inferior vena cava.

For real time RT-PCR and lung inflammatory mediator analysis, lungs were carefully removed and immediately snap-frozen and pulverized under liquid nitrogen. For assessment of total lung collagen, histological and immunohistochemical analysis, lungs were perfused with 5 ml of normal saline via the right side of the heart. For measurement of total lung collagen and RNA studies, lungs were removed, blotted dry and the trachea and major airways excised. Lungs were then weighed before being snap-frozen and pulverized under liquid nitrogen. Lungs for histological and immunohistochemical analysis were fixed following perfusion: the trachea was cannulated and lungs were insufflated with 4% paraformaldehyde in phosphate-buffered saline (PBS) at a pressure of 20 cm H_2O, followed by removal of the heart and inflated lungs *en bloc*, and immersion for 4 hours in fresh fixative. Subsequently, lungs were transferred to 15% sucrose in PBS and left overnight at 4°C, prior to transfer to 70% ethanol. For BALF analysis, the trachea was cannulated via a ventral neck incision. Normal saline was instilled in 0.5ml aliquots over 15 seconds, left *in situ* for 30 seconds, and withdrawn over 15 seconds and stored in polypropylene tubes on ice. The procedure was repeated five times and greater than 90% of the total instillate was recovered.

2.2.2. Assessment of BALF total cell number, total protein, and LDH activity

BALF cells were pelleted by centrifugation (300 g for 10 minutes at 4°C). Pellets were re-suspended in 1ml 10% FCS (Foetal Calf Serum, *Invitrogen, Paisley, UK)* in DMEM *(Dulbecco's Modified Eagle's Medium, Gibco BRL)* and cells were

counted with a standard haemocytometer. Total protein and LDH activity in BALF supernatants were assessed as per the manufacturers' protocols, using the bicinchoninic acid (BCA) protein assay *(Pierce, Rockford, IL, USA)*, and an LDH activity assay kit *(Sigma)*.

Increases in BALF protein were taken to represent vascular leak and/or loss of endothelial/epithelial barrier integrity. The BCA assay utilizes the biuret reaction, which is the reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium. Each Cu^{1+} ion then complexes with 2 molecules of bicinchoninic acid (BCA) to form a water-soluble purple-coloured reaction product which exhibits a strong absorbance at 562nm that is linear with increasing protein concentrations over a broad working range of 20µg/ml to 2000µg/ml. The amount of protein in samples can be derived by comparing sample absorbances with those of samples of known albumin concentration assayed in parallel. The assay was performed by adding 10µl of each sample or standard (in duplicate) to single wells of a 96-well microwell plate. 200µl of freshly reconstituted BCA working reagent (consisting of 50 parts reagent A and 1 part of reagent B), was added, the plate briefly agitated, and then incubated at 37°C for 30 min prior to reading the absorbance at 550nm on a Titertek Multiscan MCC/340 plate reader *(Labsystems, Finland)*.

Increases in BALF LDH activity were taken to represent cellular injury as LDH is a cytoplasmic enzyme released from cells in the context of loss of membrane integrity. The Sigma LDH activity assay is based on the reduction of NAD by LDH. The NADH formed converts a tetrazolium dye into a coloured compound which is measurable at an absorbance wavelength of 490nm. The assay was performed by adding 80µl of each sample to a single well of a 96-well microplate. Subsequently 40µl of freshly reconstituted assay mixture (consisting of equal parts of assay substrate, enzyme and dye solutions) was added. The plate was covered and incubated in the dark at room temperature for 20-30 minutes. Absorbance was then read on a Titertek Multiscan MCC/340 plate reader *(Labsystems)* at 490nm.

2.2.3. Assessment of IL-6, CCL2, TNF-α, and IL-10 levels in lung homogenates.

Lung tissue homogenates were prepared based on a method described by Keane *et al* (Keane et al. 1999a). Frozen lung powder was mixed with 0.5ml PBS

with added protease inhibitors (Compleat Mini, *Roche Diagnostics, Sussex, UK)* in polypropylene tubes. Samples were homogenised (3 x 20 seconds on ice) with a Polytron PT1035 mechanical homogeniser *(Kinematica GmbH, Litau, Switzerland)*, filtered through a 1.2μm filter *(Sartorius, Goettingen, Germany)* and aliquots were frozen at -80°C until use. IL-6, CCL2, TNF-α and IL-10 levels in homogenates were measured by ELISA, (IL-6 sensitivity >1.6pg/ml; CCL2, sensitivity >15.6pg/ml; TNF-α sensitivity >5.1pg/ml; IL-10 sensitivity >4.0pg/ml) according to manufacturer's instructions. 50μl aliquots of homogenate filtrate were analysed in duplicate. Data was expressed as pg/lung. This value was derived from the measured concentration (pg/ml) of the mediator by the following equation:

[Mediator] in lung (pg/lung) = 0.5 x ([mediator] in sample x lung wt/aliquot wt)

2.2.4. Determination of total lung collagen

Total lung collagen was determined by measuring hydroxyproline content in aliquots of pulverised lung as described previously (Campa et al. 1990),(Chambers et al. 1994),(Mutsaers et al. 1998), and assuming that lung collagen contains 12.2% w/w hydroxyproline (Laurent et al. 1981). Hydroxyproline was quantitated by reverse-phase high performance liquid chromatography (HPLC) of 7-chloro-4-nitrobenzo-oxao-1,3-diazole (NBD-Cl)-derivatised acid hydrolysates. (NBD-Cl was from *Sigma*). The basis for this assay is that the secondary amino acid hydroxyproline reacts with NBD-Cl to generate a chromophore with maximum light absorbance at 495nm. NBD-Cl also reacts with primary amino acids but these reactions occur far slower, and do not result in the generation of significant light absorbance at 495nm.

For each sample, approximately 10mg of lung powder was accurately weighed, and hydrolysed in 3ml 6M HCl for 16 hours at 110°C in a sealed pyrex glass tube. Hydrolysates were decolourised with activated charcoal, filtered through an acid-resistant 0.65μm filter *(Millipore ltd., Watford, UK)*, and diluted 1 in 100. A 200μl aliquot of this diluted hydrolysate was then dried down by centrifugal vacuum concentration using a SPD131DDA Speedvac *(Thermo Electron Corporation, Cambridge, UK).* The resulting residue was re-dissolved in 100μl HPLC grade H_2O, buffered with 100μl 0.4M potassium tetraborate (pH 9.5) *(Sigma)* and reacted with

100µl 36mM NBD-Cl (in methanol). Samples were wrapped in aluminium foil to protect them from light and incubated in a water bath at 37°C for 20 minutes. The reaction was then stopped by the addition of 50µl 1.5M HCl. At this point 150µl of 3.33x Buffer A was also added (buffer A is described in **table 2.2**). Samples were then filtered through a 0.22µm filter *(Millipore)* into a polypropylene insert within an Amber Snap Seal vial *(Laboratory Sales ltd., Rochdale, UK)*. These vials were then loaded onto the HPLC autosampler and the samples were sequentially injected onto the column by an automated injector.

The HPLC apparatus employed for these measurements was a Beckman System Gold HPLC *(Beckman Coulter, Bucks, UK)* with a reverse-phase cartridge column (LiChroCART LiCrospher 250mm length x 4mm diameter, 5µm particle size, 100 RP-18; *BDH/Merck, Lutterworth, UK)* protected by a directly coupled pre-column (LiChrosorb, 4mm x 4mm, 5µm, 100 RP-18; *BDH/Merck*). Columns were continuously maintained at 40°C in a heated column oven. Immediately prior to injection of samples onto the HPLC column, all running buffers were degassed, and the HPLC system equilibrated in running buffer A for 40 minutes.

Standards of 50pmol/L hydroxyproline *(Sigma)* were included among the samples to enable calibration and quantitation. NBD-Cl derivatives in samples and standards were eluted with an acetonitrile gradient, which was achieved by changing the relative proportions of running buffers over time. Conditions and buffers employed in this process are shown in **table 2.2.**

Column	**LiChrosopher, 100 RP-18, 250 x 4mm, 5μm**	
Mobile phase	***Buffer A*** - aqueous acetonitrile (8% v/v) 50mM sodium acetate, pH 6.4 ***Buffer B*** - aqueous acetonitrile (75% v/v)	
Column flow rate	1.0 ml/min	
Column temperature	40°C	
Wavelength for detection	495nm	
Elution gradient	Time (min)	% Buffer B
	0	0
	5	5
	6	80
	12	80
	12.5	0
	25	0

Table 2.2. Conditions and buffers for the separation of hydroxyproline by reverse-phase HPLC

Eluted amino acids were detected by monitoring absorbance at 495nm. Hydroxyproline elutes as a discrete peak between 5 and 7 minutes following its injection onto the column. Quantitation of the hydroxyproline content in each sample was determined by comparing peak areas of the hydroxyproline peak of each sample with those of the standards of known hydroxyproline concentration.

Total lung collagen was expressed as mg/lung and derived from the equation:

Total lung collagen =

peak area x 10 x 100 x 5 x 1/1000 x 100/12.2 x 131/1000000 x lung weight/powder weight

2.2.5. Histological analysis

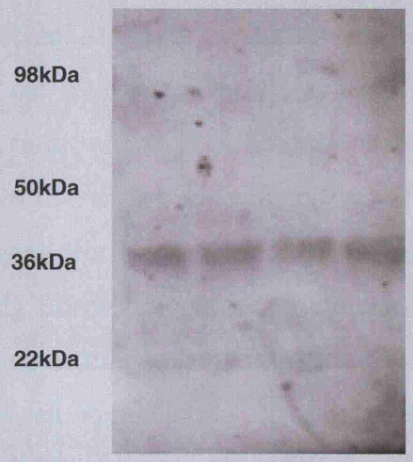
Individual lobes of mouse lungs were placed in processing cassettes, dehydrated through a serial alcohol gradient, and embedded in paraffin wax blocks. Blocks were cooled to 4°C and 4μm sections were cut using a microtome. Prior to immunostaining, these 4μm thick lung tissue sections were dewaxed in xylene, rehydrated through decreasing concentrations of ethanol, and washed in PBS.

2.2.5.1. Immunohistochemistry for CTGF, TGF-β_1, PAR_1, and CCL2

For CTGF, TGF-β_1, and PAR_1 immunostaining, antigens were unmasked by microwaving sections in 10mM citrate buffer, pH 6.0 (2 x 10 min). For CCL2 immunohistochemistry, antigen unmasking was found not to be useful in prior optimization studies, and therefore was omitted. Immunostaining was undertaken using the avidin biotinylated enzyme complex method *(Vector Laboratories, Burlingame, CA, USA)* with antibodies against CTGF *(Santa Cruz)* at a concentration of 0.4 μg/ml, TGF-β_1 *(Santa Cruz)* at a concentration of 1 μg/ml, SFFL (PAR_1) at a concentration of 0.14 μg/ml, and CCL2 *(Santa Cruz)* at a concentration of 0.5μg/ml with equivalent concentrations of polyclonal non-immune IgG controls. After incubation with the appropriate biotin-conjugated secondary antibody (all from Dako, *Glostrup, Denmark)*, and subsequently streptavidin-HRP solution *(Dako)*, colour development was performed using 3,3'-diaminobenzidine tetrahydrochloride (DAB, *Vector Laboratories*) as a chromogen. Sections were counterstained using Gill-2 haematoxylin *(Thermo-Shandon, Pittsburgh, PA, USA)*, dehydrated through a series of incubations in increasing concentrations of ethanol and then xylene, and mounted using a Sakura Coveraid automatic coverslipping machine and Tissue-Tek coverslipping film *(Bayer Diagnostics, Basingstoke UK)*. Specificity of the signal obtained was confirmed by showing that no positive staining was detectable when the primary antibody was substituted with an equivalent concentration of non-immune polyclonal IgG, or when it was omitted altogether. In addition, because there were no published reports of the use of the *Santa Cruz* L-20 CTGF antibody in immunostaining of bleomycin-injured murine lung, a western blot of lung

homogenates was performed (**figure 2.1**) to demonstrate that this antibody reacted with a species of approximately the correct size of CTGF (38kDa). Homogenates were prepared as already described. Western blotting methods employed in this work are described in detail later in this chapter (**Please see section 2.3.**)

Figure 2.1. Western blot of mouse lung homogenates for CTGF. A single immunoreactive band with an approximate size of 38kDa, consistent with CTGF, was obtained



2.2.5.2. Semi-quantitative image analysis of CTGF and TGF-β_1 immunostaining

A semi-quantitative image-analysis technique was used to evaluate the extent of immunostaining for these two proteins per lobe using a JVC KY-F55BE digital camera *(JVC, Yokohama, Japan)* and KS300 software *(Carl Zeiss Vision GmbH, Eching, Germany)*. The colour intensity representative of positive immunostaining following saline or bleomycin instillation was established after visual inspection of all sections, and a threshold defined. Individual lobes were examined blind to experimental group (x 100 magnification) using an Olympus BX40 microscope *(Olympus, London, UK)*. For each field of view, the percentage of area staining of greater intensity than the defined threshold was taken to represent the percentage of

positive immunostaining. For each lobe, 2 separate non-overlapping fields of view, which appeared to be the most positively stained, were chosen. They were discarded if non-representative areas such as airway epithelium occupied greater than 15% of the field of view, or fields exhibited obvious staining artefact or overlapped the pleural surface. The data is expressed as mean percentage of positive immunostaining per lobe.

2.2.5.3. Histological evaluation of lung architecture

Two different staining methods were used to evaluate lung architecture. A modified trichrome staining method was used to assess lung fibrosis. This method demonstrates all tissue elements differently based on the use of differring permeability of tissues to dyes of varying molecular size. The large Chicago Sky Blue 6GX dye permeates collagen fibrils causing areas of fibrosis to appear blue. However the pictures depicting fibrosis displayed in the results section of this thesis are of a low power magnification and are not intended to enable visualization of collagen, but rather to enable an appreciation of gross lung architecture. In order to enable appreciation of inflammatory cell infiltration in elderly mice, a heamatoxylin and eosin method was used. Automated staining of sections was performed using a Sakura DRS 601 Diversified Stainer *(Bayer Diagnostics)*. Solutions and incubation times through which the sections were processed are shown in **table 2.3** (modified Trichrome), and **table 2.4** (H&E).

Step No:	Time:	Solution:
1	3 min	Xylene
2	3 min	Xylene
3	2 min	100% Industrial Methylated Spirit (IMS)
4	2 min	90% IMS
5	2 min	70% IMS
6	5 min	Lugol's iodine
7	3 min	Sodium Thiosulphate
8	1 min	Wash in H_2O
9	10 min	Celestine Blue
10	1 min	Wash in H_2O
11	30 secs	Distilled H_2O (dH_2O)
12	5 min	Haematoxylin
13	30 secs	Wash in H_2O
14	20 secs	Acid Alcohol (1% HCl in 70% ethanol)
15	30 secs	Wash in H_2O
16	2 min	Wash in H_2O
17	30 secs	90% IMS
18	8 mins	Orange G (0.2% in Picric alcohol (100% ethanol saturated with picric acid))
19	5 secs	dH_2O
20	7 min	Red Mix (Ponceaux 2R 0.5% & Acid Fuchsin 0.5% in 1% Glacial acetic acid)
21	20 secs	dH_2O
22	30 secs	Phosphotungstic acid (1% in AD)
23	20 secs	dH_2O
24	80 secs	0.5% Chicago SkyBlue 6BX in 1% glacial acetic acid
25	20 secs	1% glacial acetic acid
26	20 secs	70% IMS
27	20 secs	90% IMS
28	1 min	100% IMS
29	1 min	Xylene
30	1 min	Xylene
31	2 min	Xylene

Table 2.3: Solutions and incubation times for modified Trichrome staining

Step No:	Time:	Solution:
1	3 min	Xylene
2	3 min	Xylene
3	2 min	100% Industrial Methylated Spirit (IMS)
4	2 min	90% IMS
5	2 min	70% IMS
6	2 min	50% IMS
7	1 min	dH_2O
8	5 min	Haematoxylin
9	10 sec	Wash in H_2O
10	10 sec	Wash in H_2O
11	08 sec	Acid alcohol
12	30 secs	Wash in H_2O
13	2 min	Wash in H_2O
14	6 min	Eosin
15	15 secs	Wash in H_2O
16	30 secs	50% IMS
17	45 secs	70% IMS
18	1 min	90% IMS
19	1 min	100% IMS
20	2 min	100% IMS
21	1 min	Xylene
22	2 min	Xylene
23	2 min	Xylene

Table 2.4: Solutions and incubation times for H&E staining

(Reagents used in these staining protocol were obtained from the following suppliers: Acid Fuchsin, Haematoxylin, IMS, Xylene, Phosphotungstic acid, potassium iodide: *BDH*; Picric acid, sodium thiosulphate, iodine, Celestine Blue, Chicago Sky Blue 6BX, Ponceau 2R: Sigma; Orange G: *Raymond A Lamb, Eastbourne, Sussex, UK*; Glacial acetic acid: *Fisher Scientific, Loughborough, Leicestershire, UK*).

2.2.5.4. Histological evaluation of fibrin

In order to demonstrate fibrin fibres, a modified staining technique, based on that of Lendrum *et* al (LENDRUM et al. 1962) and involving a Ponceaux de Xylidine and Acid Fuchsin mix, was employed. Solutions and incubation times through which the sections were processed are as shown in **table 2.5**.

Step No:	Time:	Solution:
1	3 min	Xylene
2	3 min	Xylene
3	2 min	100% Industrial Methylated Spirit (IMS)
4	2 min	90% IMS
5	2 min	70% IMS
6	5 min	Lugol's iodine
7	3 min	Sodium Thiosulphate
8	1 min	Wash in H_2O
9	10 min	Celestine Blue
10	1 min	Wash in H_2O
11	30 secs	Distilled H_2O (dH_2O)
12	5 min	Haematoxylin
13	30 secs	Wash in H_2O
14	20 secs	Acid Alcohol (1% HCl in 70% ethanol)
15	30 secs	Wash in H_2O
16	2 min	Wash in H_2O
17	30 secs	90% IMS
18	8 mins	Orange G (0.2% in Picric alcohol (100% ethanol saturated with picric acid)
19	5 secs	dH_2O
20	7 min	Red Mix (Ponceaux 2R 0.5% & Acid Fuchsin 0.5% in 1% Glacial acetic acid)
21	20 secs	dH_2O
22	30 secs	Phosphotungstic acid (1% in AD)
23	20 secs	dH_2O
24	20 secs	50% IMS
25	20 secs	70% IMS
26	20 secs	70% IMS
27	20 secs	90% IMS
28	1 min	100% IMS
29	1 min	Xylene
30	1 min	Xylene
31	2 min	Xylene

Table 2.5: Solutions and incubation times for histological demonstration of fibrin.

2.2.6. Real Time RT-PCR analysis of lung tissue for CTGF and TGF-β_1 mRNA levels

Total RNA was isolated from frozen powdered lung tissue with TRIzol reagent *(Gibco-BRL)* as per the manufacturer's instruction. TRIzol is a solution of phenol and guanidine isothiocyanate. It effectively disrupts cells and dissolves cell components, whilst maintaining the integrity of RNA. Following addition of chloroform and centrifugation the disrupted cell suspension separates into upper aqueous and lower

organic phases. RNA remains exclusively in the aqueous phase and is precipitated from it by the addition of isopropanol.

RNA pellets were redissolved in 17µl DEPC H2O *(Ambion, Cambridge, UK)*, and this RNA solution was DNase treated with DNA-free *(Ambion)* as per the manufacturers instruction to remove contaminating genomic DNA. Optical density of samples was assessed on an Ultrospec 3000 spectrophotometer *(Amersham Biosciences, Bucks, UK)*, and the quantity of RNA in each sample derived from the absorbance at 260nm. 0.5-1µg RNA from each sample was then mixed with running buffer containing ethidium bromide, and ran on a 1% agarose formaldehyde gel. Images of the RNA were captured with a Syngene Gene Genius Bio-imaging system *(Synoptics, Cambridge, UK)*. A ratio of approximately 2:1 of intensities of the 28S (upper band) to the 18S (lower band) confirmed that the RNA was not significantly degraded.

Subsequent steps (as described hereafter) were undertaken by Dr Bin Shan of Tulane University, New Orleans, USA as part of an ongoing collaboration between Dr Rachel Chambers and Professor Joseph Lasky. Reverse transcription (RT) was carried out with 2 µg of total RNA in a reaction volume of 20 µl using Cloned AMV First-Strand cDNA Synthesis Kit (*Invitrogen, Carlsbad, CA, USA*) following the provided instructions. Oligo $(dT)_{20}$ was used as the primer for RT. Real-time PCR was conducted using iQ SYBR Green Supermix on an iCycler iQ Real-time Detection System following the manual and analysed using iCycler iQ Real-time PCR Detection System Software Version 3.0A (*all Bio-rad, Hercules, CA, USA*). Cycling conditions were optimized such that the PCR efficiency was 100% (±5%).

For CTGF each cycle was 95°C, 30 seconds; then 58°C, 30 seconds. For TGF-β_1 each cycle was 95°C, 15 seconds; then 64°C, 45 seconds. For 36B4, each cycle was 95°C, 20 seconds; then 57.5°C, 30 seconds. The specificity of the PCR product was confirmed by melting curve analysis and gel electrophoresis. Single products of the expected size were obtained: 36B4: 109bp; CTGF: 232bp; TGF-β1: 155bp. A detailed description of the basis of calculation of relative mRNA abundance is described later in this chapter (**please see section 2.3.3.7**). Briefly, crossing point (Cp) values were derived from the amplification plots. CTGF mRNA levels in each sample were normalized for 36B4 using the equation $CTGF/36B4=2^{\wedge Cp(36B4)-Cp(CTGF)}$, allowing direct comparison between samples. Similarly, an equivalent equation was used to

derive TGF-β_1 mRNA levels. The lowest mean value was then assigned an arbitrary value of 1 and the values of the other samples scaled accordingly.

2.3. Epithelial cell culture studies

2.3.1. Epithelial cells and experimental conditions

The MLE-15 cell line was originally generated by Wikenheiser *et al* (Wikenheiser et al. 1993). Spontaneously occurring tumours were isolated from transgenic mice harbouring the SV40 T antigen under the control of the human SP-C promoter region, and colonies of epithelial cells were allowed to grow out from minced tumour tissue. MLE-15 represents a clonal cell line derived in this way. These cells display features of type II lung alveolar epithelial cells, namely: polygonal morphology, microvilli, cytoplasmic multivesicular bodies and multilamellar inclusion bodies, and expression of SP-A and SP-B mRNA. These cells maintain this epithelial phenotype for 30-40 passages in culture. MLE-15 cells require culturing in HITES medium which contains a number of growth factors and hormones necessary to support their growth and phenotype. For experimental work described in this thesis, MLE-15 cells were maintained in HITES medium consisting of RPMI 1640 *(Gibco-BRL)* supplemented with 2% Foetal Calf Serum (FCS), 5μg/ml insulin, 10μg/ml transferrin, 30nM sodium selenite, 10nM hydrocortisone, 10nM β-estradiol, 4mM L-glutamine, 10nM HEPES, and 200units/ml penicillin/streptomycin (all *Sigma*).

The A549 cell line is a continuous cell line derived from lung carcinomatous tissue. They retain many but not all phenotypic features characteristic of primary human type II alveolar epithelial cells, including cuboidal type II-epithelial like morphology (Foster et al. 1998), apical microvilli (Alink et al. 1980), the presence of intracellular lamellar bodies (Foster et al. 1998),(Shapiro et al. 1978), the expression of pro-surfactant protein C (pSP-C) (Vaporidi et al. 2005), the synthesis of phospholipids (Shapiro et al. 1978), and the ability to form polarised cell monolayers *in vitro* (Foster et al. 1998). For experimental work described in this thesis, A549 cells were maintained in F12K *(Gibco-BRL)* with 10% FCS, 200 units/ml penicillin/streptomycin and 4mM L-glutamine.

The BEAS-2B cell line was originally generated by isolating epithelial cells from normal human bronchial epithelium, infecting those cells with an adenovirus 12-SV40 virus hybrid, and then cloning them. Some investigators maintain BEAS-2B in a serum-free medium supplemented with growth factors and hormones because of concerns that serum may promote squamous differentiation of these cells, just as it

may in primary bronchial epithelial cells. However, a number of the supplements required when serum is absent have previously been demonstrated to affect the production of pro-inflammatory and pro-fibrotic mediators by epithelial cells *in vitro*. Furthermore such effects involve specifically those mediators being examined in this thesis. For instance, amongst the recommended supplements for BEAS-2B culture: the epidermal growth factor family of cytokines may modulate expression of CCL2 (Mascia et al. 2003) and CTGF (Wenger et al. 1999); glucocorticoids can influence expression of CCL2 (Natori et al. 1997) and CTGF (Matsuda et al. 2005); insulin can influence expression of CCL2 (Mamounas et al. 1991) and TGF-β (Romberger et al. 1995); transferrin can influence expression of CCL2 (Wang et al. 1997); and retinoic acid can influence expression of CTGF (Freemantle et al. 2002) and TGF-β (Niles et al. 1994). Therefore, where BEAS-2B were used for experiments outlined in this thesis, they were maintained in a basic buffered medium with essential nutrients, 10% foetal calf serum, and without specific growth factor, steroid, or hormone supplements. Under similar conditions, BEAS-2B have been shown to retain electron microscopic features of epithelial cells, and express cytokeratin 18: a marker of columnar epithelium and not of squamous epithelium (Zhu et al. 1999). For experimental work described in this thesis, BEAS-2B cells were maintained in RPMI 1640 with 10% FCS, 200 units/ml penicillin/streptomycin and 4mM L-glutamine.

Epithelial cell lines used for *in vitro* experiments were between passages 4-8 for MLE-15 cells, and between passages 12-25 for A549, BEAS-2B, HFL-1 and MLF cells. The HFL-1 (human foetal lung fibroblast) cell line was used as a PAR_1-expressing positive control in some experiments. MLE-15, A549 and BEAS-2B cells were incubated in a humidified atmosphere containing 5% CO_2. HFL-1 and MLF were maintained in DMEM *(Gibco-BRL)* with 10% FCS at 37°C in a humidified atmosphere containing 10% CO_2.

Cells were propagated in $75cm^2$ or $175cm^2$ culture flasks. Upon reaching visual confluence, the medium was aspirated, and the cell layer washed with 10ml PBS. This was then aspirated and trypsin (0.05% w/v)/ EDTA (0.02% w/v) *(Gibco-BRL)* was added (2ml for a $75cm^2$ flask; or 4ml for a $175cm^2$ flask). Flasks were incubated at 37°C until cell detachment from the tissue culture plasticware was observed by inspection using inverted microscopy. Trypsin was then neutralised by the addition of 10ml FCS. The cell suspension was aspirated and pelleted by

centrifugation at 300g for 5 minutes at room temperature. Cells were resuspended in fresh medium with the appropriate supplements and FCS. Cell suspensions were then either divided 1 in 4 and added to fresh flasks or alternatively adjusted to the appropriate seeding density (using a haemocytometer) and plated for experiments. Volumes of media in flasks were kept at 20ml in $75cm^2$ flasks, and 40ml in $175cm^2$ flasks. For experiments, cells were seeded at 2×10^5 cells/ml in 6-well plates (2ml per well) or 12 well plates (1ml per well), and grown until they reached 90% confluency at which point they were washed in PBS and then quiesced for 24 hours in serum-free basic medium without supplements prior to their use in experiments.

Primary human type II alveolar epithelial (ATII) cells were isolated from lung of grossly normal appearance obtained following resection for lung carcinoma. This was undertaken by Dr Andrew Thorley (NHLI, London) with approval of the Royal Brompton and Harefield Hospital Ethical Committee. Tissue was perfused with sterile 0.9% sodium chloride and inflated with trypsin (0.25% in HBSS (HEPES-buffered salt solution)), and incubated at 37°C for 45 minutes; trypsin was replaced twice during this time. The tissue was finely chopped in the presence of newborn calf serum (NCS). The chopped tissue was then incubated with DNase (250µg/ml) and the mixture passed through a 400µm filter, followed by a 40µm filter to remove large tissue debris. The cell suspension was then centrifuged at 1300 rpm (290g) for 10 minutes and the resulting pellet re-suspended in DCCM-1 media containing 50µg/ml DNase. The cells were incubated in tissue culture flasks for two hours at 37°C in a humidified incubator to allow differential adherence and removal of contaminating mononuclear cells.

After two hours, non-adherent ATII cells were removed by aspirating the media and the cell suspension centrifuged at 1300 rpm (290g) for 10 minutes. The cell pellet was then re-suspended in DCCM-1 containing 10% NCS and 1% penicillin-streptomycin-glutamine (PSG) at a concentration of 1×10^6 cells/ml. Cells were then seeded at 1×10^6 ATII cells per well on plates that had previously been coated with 300µl of a 1% solution of Vitrogen-100. Cells reached confluence by 48 hours. Even spread and distribution of monolayers was confirmed under light microscopy. Monolayers were also demonstrated to stain uniformly for alkaline phosphatase activity, a marker of an epithelial cell phenotype. Confluent monolayers of ATII cells

were quiesced for 24 hours in serum-free basic medium without supplements prior to their use in experiments.

For experiments involving the PAR_1 antagonist RWJ-58259, and the protein kinase C inhibitor Ro-31-8425, these compounds were pre-incubated for 20 minutes with cells in fresh medium, and the concentration of DMSO vehicle was standardised so that all samples contained the same concentration of DMSO. Subsequently thrombin or factor Xa *(Calbiochem)* or TFLLR-NH_2 of the required concentrations were added to reach the desired final concentration.

Where factor Xa was used in experiments, it was at a concentration of 25nM as it is unlikely that higher concentrations are physiologically relevant within the lung *in vivo*, based on the observations that the plasma concentration of the zymogen factor X is only 170nM (Halkier.T 1991), and the amount of factor Xa in BALF of injured rat lung has been reported to be only 1.4nM (Chan et al. 2006). The circulating concentration of prothrombin is around 1000nM, but a physiologically relevant concentration (in the lung) of thrombin is probably around 10nM-25nM, based on the observation of BALF concentrations of around 6nM in healthy volunteers, and 13nM in systemic sclerosis patients (Hernandez-Rodriguez et al. 1995).

2.3.2. *In vitro* models of lung epithelial cell responses

Results outlined in chapters 2 and 3 of this thesis are those obtained using cultured cells, in particular: the transformed murine distal lung epithelial cell line: MLE-15, the transformed human lung bronchial epithelial cell line: BEAS-2B, and the transformed human lung alveolar epithelial cell line: A549. Primary human lung alveolar epithelial cells have also been used where possible.

There are specific and distinct limitations to *in vitro* experiments using both primary cells, and transformed cell lines. Primary cells are more difficult to obtain, and tend to have more stringent requirements for maintenance *in vitro*. When grown *in vitro*, primary type II alveolar epithelial cells have an inherent propensity to lose characteristic phenotypic features such as apical microvilli (Diglio & Kikkawa 1977), lamellar body inclusions, production of phospholipids (Mason et al. 1977), and synthesis of surfactant proteins (Shannon et al. 1992). When grown on tissue culture plastic, these cells progressively adopt a type I alveolar epithelial cell-like phenotype (Danto et al. 1992),(Rannels et al. 1989), although manipulation of the culture

conditions can largely prevent this process. For instance, culturing cells on plastic coated with laminin and collagen (Olsen et al. 2005), or on a floating collagen gel above a fibroblast feeder layer (Rannels et al. 1989) both help maintain morphological features of type II alveolar epithelial cells.

The limited availability of such primary cells made it largely impractical to attempt a programme of *in vitro* research using exclusively primary human lung epithelial cells. Conversely cell lines are readily available, can be passaged a number of times without significant phenotypic change, and tend to have less stringent requirements for maintenance *in vitro*. However they may retain fewer phenotypic features of their cell type of origin than primary cells.

All *in vitro* studies have limitations, whether they involve transformed cell lines or primary cells. In such studies, biological responses are being examined out of context, in that the normal interactions with neighbouring cells and biological mediators, and the precise physiological conditions present *in vivo* cannot be replicated adequately *in vitro*. Therefore an *in vitro* experiment at best shows that a certain cell type has the potential to respond similarly *in vivo* to how it does *in vitro*. Nonetheless, experiments using cultured cells play a fundamental and important part of characterisation of biological responses and mechanisms of disease.

2.3.3. Assessment of epithelial cell gene expression by *(in-house)* real-time RT-PCR studies

2.3.3.1. RNA isolation

Cells were maintained in 6-well plates for RNA studies. Cell layers were lysed by the addition of 1ml TRIzol *(Gibco-BRL)* per well, and RNA extracted as per the manufacturers instruction. RNA pellets were redissolved in 17µl DEPC H_2O *(Ambion, Cambridge, UK)*, and this RNA solution was DNase treated with DNA-free *(Ambion)* as per the manufacturers instruction to remove contaminating genomic DNA. Optical density of samples was assessed on an Ultrospec 3000 spectrophotometer *(Amersham Biosciences, Bucks, UK)*, and the quantity of RNA in each sample derived from the absorbance at 260nm. 0.5-1µg RNA from each sample was then mixed with running buffer containing ethidium bromide, and ran on a 1% agarose formaldehyde gel.

Images of the RNA were captured with a Syngene Gene Genius Bio-imaging system *(Synoptics, Cambridge, UK)*. A ratio of approximately 2:1 of intensities of the 28S (upper band) to the 18S (lower band) confirmed that the RNA was not significantly degraded.

2.3.3.2. cDNA synthesis

cDNA was prepared from 1μg of RNA using an RNA PCR Core kit *(Applied Biosystems, CA, USA)*, and reactions were performed according to the manufacturer's instructions. Briefly, the following mix was prepared, and incubated at room temperature for 10 minutes, 42°C for 15 minutes, 99°C for 5 minutes, then 5°C for 5 minutes:

4 μl MgCl
2 μl 10x PCR Buffer
8 μl dNTP mix
1 μl random hexamers
3 μl RNA (equivalent to 1μg)
1 μl RNase Inhibitor
1 μl reverse transcriptase

2.3.3.3. Primer design

The primers were designed using internet based programmes. The Ensembl database (http://www.ensembl.org/) was used to locate the genomic and RNA accession codes. Spidey (http://www.ncbi.nlm.nih.gov/IEB/Research/Ostell/Spidey/) was used to align the genomic and RNA sequences in order to locate intron/exon boundaries. Primers were designed to span introns, eliminating the possibility of amplifying genomic DNA. The mRNA sequence was copied into the primer-designing software, Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The parameters were set at: product size 85-130 bp; primer size 18-22 nucleotides long; primer melting temperature 58°C to 62°C with an optimum of 60°C and a maximum temperature difference of 0.5°C; primer GC % was

40 % to 60 % with an optimum of 50 %; maximum self-complimentarity was set at 6.0 and maximum 3' self-complimentarity of 2.0 and finally, the maximum poly-X was set at 4 to avoid runs of nucleotides. The primers were then selected from the list and run *in silico* using FastPCR software. A BLAST search (http://www.ncbi.nlm.nih.gov/BLAST) was also performed to check that the forward and reverse primers were specific for the intended sequence. Primers were subsequently manufactured by *Invitrogen (Paisley, UK).* In this thesis, real-time RT-PCR was used to assess gene expression of murine and human PAR_1, CCL2, CTGF and TGF-β_1 in cultured cells.

2.3.3.4. Real-time RT-PCR

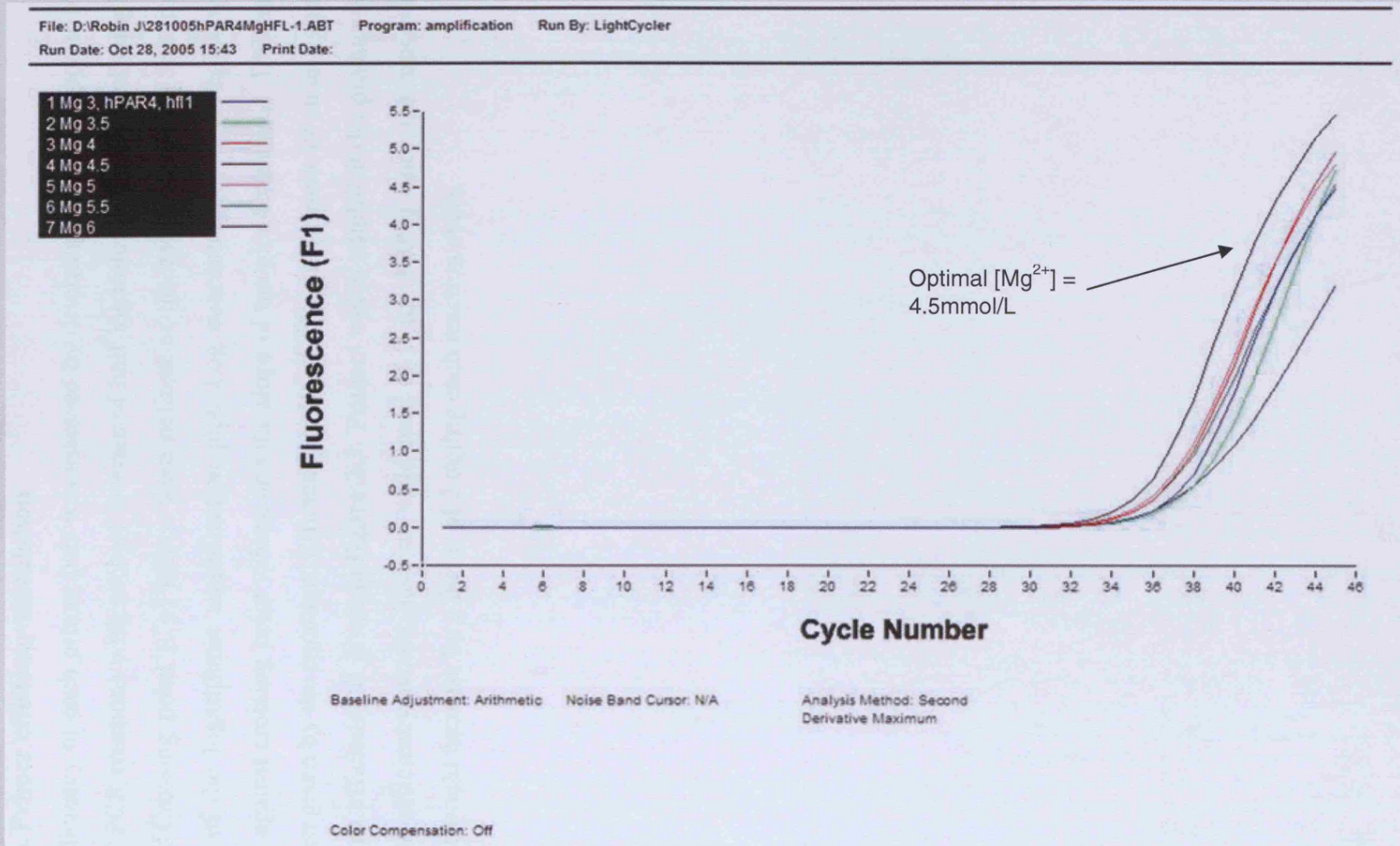
Real time RT-PCR was conducted using the Platinum SYBR Green qPCR SuperMix, with 1ng of cDNA and forward and reverse primers each at a final concentration of 500nM, on a Roche LightCycler 1.5 and analysed using LightCycler Real-time PCR Detection System Software Version 3.5 (all from *Roche, Sussex, UK*). Cycling conditions were: 2 min at 50°C (one cycle), 2 min at 95°C (one cycle); then 5 secs at 95°C, 5 secs at 55°C, and 15 secs at 72°C (45 cycles). In this system the measurable fluorescence intensity directly correlates with the amount of PCR product formed. The more abundant the product, the earlier (i.e. the lower the cycle number) the increase in fluorescence is detected. Whenever a new pair of primers was used for the first time, a non-template control PCR reaction involving these primers, but in the absence of cDNA, was set up to ensure product was not formed in the absence of template cDNA.

2.3.3.5. Optimisation of magnesium concentration for each primer pair

Prior to the use of primers in analysis of mRNA levels in experimental samples, their optimal magnesium concentration was established. This was achieved by performing a number of RT-PCR reactions in parallel, each with template cDNA from a sample known to express the gene targeted by the primers, but with differing concentrations of Mg^{2+} in the SYBR Green PCR supermix. The optimal Mg^{2+} concentration was chosen on the basis that it was the one associated with the steepest logarithmic amplification plot, and typically also the lowest cycle number at which

detectable amplification occurred. This was the concentration subsequently always employed for this primer pair. An example of a Mg^{2+} titration is shown in **figure 2.2.**

Figure 2.2. Magnesium titration for human PAR_4 primers using cDNA from HFL-1 fibroblasts as a template. The optimal magnesium concentration for these primers is 4.5 mmol/l

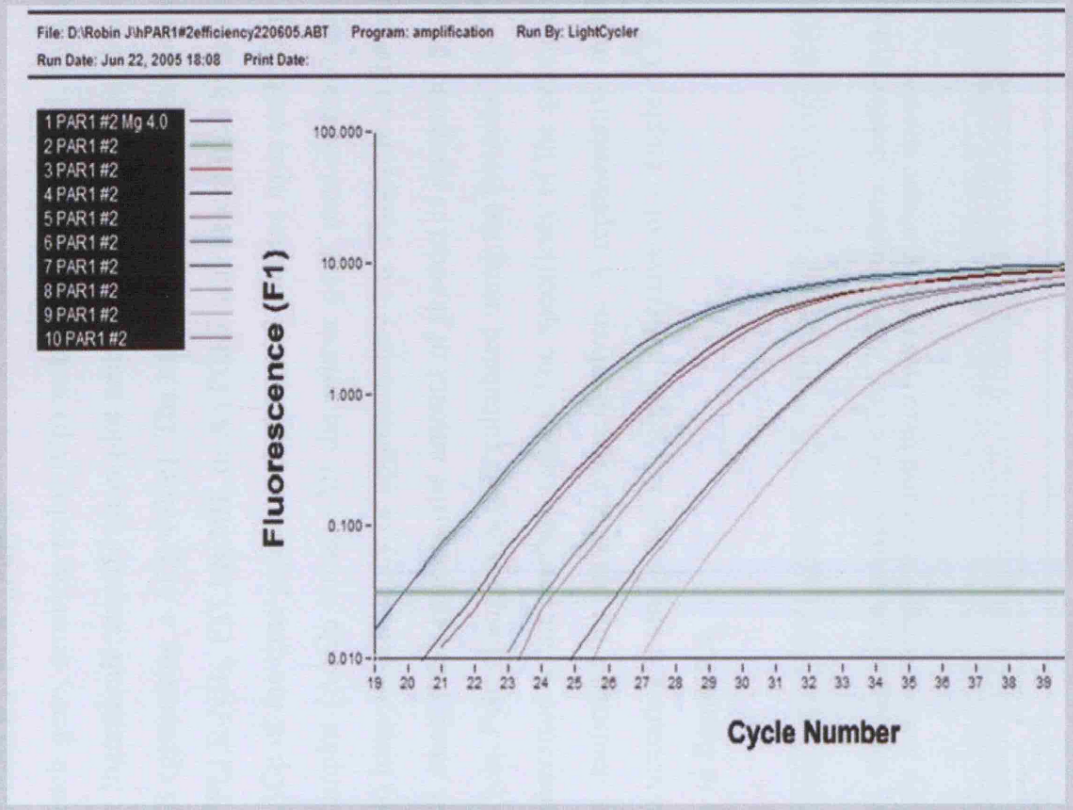


2.3.3.6. Primer efficiency calculation

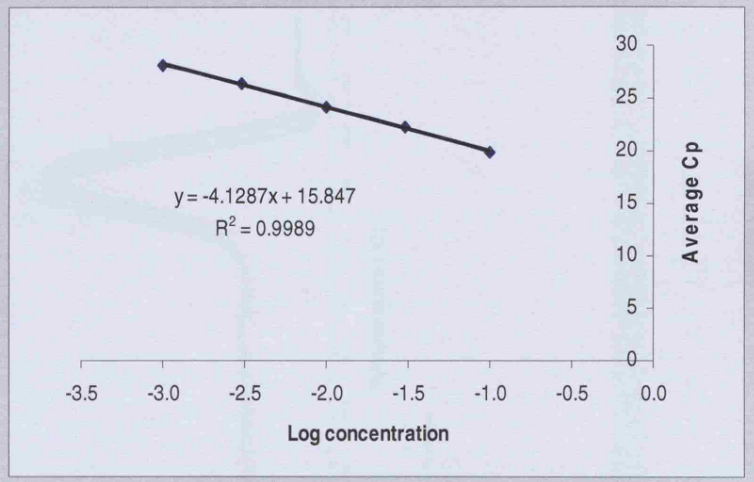
The efficiency of each primer pair was assessed by determining crossing points (Cp) for RT-PCR reactions with each of a series of half logarithmic dilutions of template cDNA. Crossing point (Cp) values were defined as the earliest point of the linear region of the logarithmic amplification plot. Log concentrations of samples were plotted against crossing point values, and the slope of the plot determined. Efficiency was then given by the equation: Efficiency = $10^{\wedge(-1/\text{slope})}$. An example of an assessment of primer efficiency is given in **figure 2.3**. Primers which amplified the product with absolute efficiency would have an efficiency of 2 (or 100%) since the amount of product would increase by a factor of 2 during each reaction cycle.

Figure 2.3. Efficiency calculation for human PAR_1 primers, using half-logarithmic dilutions of cDNA (ran in duplicate) from A549 epithelial cells. (a) amplification plot. (b) plot of log concentration against Cp values. Efficiency = 1.75

(a)



(b)

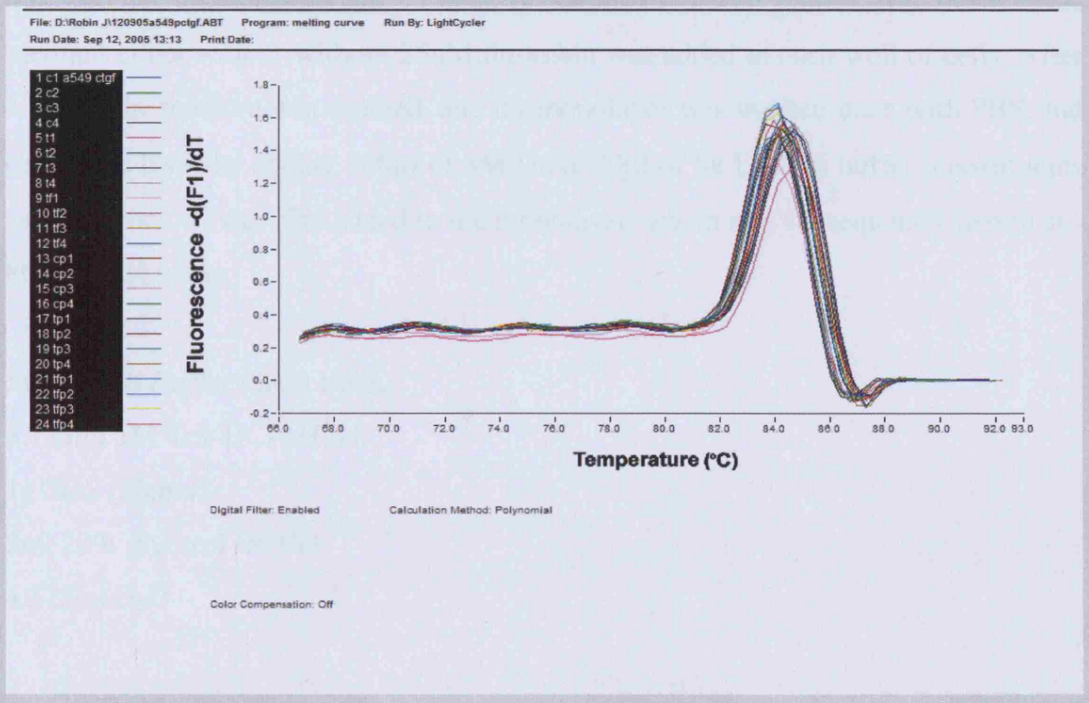


Efficiency = 10^(-1/-4.13) = 1.75

2.3.3.7. Real-time RT-PCR analysis

For each gene, crossing point (Cp) values were determined from the linear region of the logarithmic amplification plot and normalised by subtraction of the Cp value for 18S (generating a ΔCp value). The response to stimulation with thrombin, Factor Xa, TFLLR-NH_2, FTLLR-NH_2 or AYPGKF was determined by subtraction of the average ΔCp of the time-point matched control samples from each ΔCp value for stimulated samples (ΔΔCp value). All the primer pairs used in real-time RT-PCR were found to have an efficiency of approximately 1.8. Therefore in the exponential phase of PCR using these primers, the amount of product is increased by a factor of 1.8 in each cycle. Fold change was thus calculated using the formula $1.8^{\wedge -\Delta\Delta Cp}$ (where ΔΔCp was converted to an absolute value). The specificity of the products obtained by PCR was confirmed by melting curve analysis. A representative melting curve analysis demonstrating a single melting point indicative of a single PCR product is shown below in **figure 2.4**.

Figure 2.4. Melting curve analysis of CTGF PCR products, confirming products have a single melting temperature and thus represent a single species.



2.3.4. Human CCL2 ELISA

The initial optimisation of this ELISA was already established in the laboratory by Dr Paul Mercer (Centre for Respiratory Research, UCL). Each well of a 96 well ELISA plate was coated with 50µl 2µg/ml MAB679 *(RnD systems)*, serving as a capture antibody, in bicarbonate coating buffer, left for 5 hours at room temperature, and then washed. 100µl of PBS/1% BSA was added to each well and the plate was left overnight at 4°C. 50µl of either CCL2 standard *(Peprotech)* or sample was added, and the plate was incubated for 2 hours at room temperature (RT). Following washing, 50µl BAF279 (100ng/ml) *(RnD systems)*, serving as a biotinylated detector antibody, was added to each well, and left for 2 hours at RT. The plate was washed, and 50µl of 2µg/ml dilution of streptavidin-HRP *(Dako)* was added for 30 minutes. After washing, 50µl TMB substrate *(BD Pharmingen)* was added to each well. After 30 minutes, colour development was quenched with 0.19M H_2SO_4, and absorbances were measured at 450nm.

2.3.5. CTGF western blotting

Medium was aspirated from monolayers of A549 cells which had been quiesced for 24 hours in the absence of serum in 6 well plates. 1ml fresh F12K medium either with or without 25nM thrombin was added to each well of cells. After 4 hours, the medium was drained, and the monolayer was washed once with PBS, and cells were lysed by adding 100µl of 8M Urea. 20µl of 5x Laemlli buffer (constituents as shown below) was also added to the monolayer, which was subsequently frozen at -80°C for 24 hours.

5x Laemlli Buffer (5ml) stock:

3.125ml 1M Tris HCl pH 6.8

1g SDS *(Sigma)*

2ml 20% glycerol *(BDH)*

4.375ml H_2O

Cell lysates were collected by scraping with a rubber policeman at 4°C. 6µl of 2-β-mercaptoethanol was then added to each sample (to a final concentration of 5%).

Samples were boiled for 10min at 96°C, pulse-spun down briefly on a table-top centrifuge (10 seconds). Either 30µl of sample or 5µl of SeeBlue® Plus 2 protein ladder *(Invitrogen)* was then added to each well of a 10-well acrylamide gel.

This gel consisted of a 10% acrylamide separating gel and a 4% stacking gel. Their constituents are as shown below. The separating gel was allowed to set in a 1mm gel cast cassette *(Invitrogen)*. Once set, the stacking gel was poured over the separating gel, and a 10-well comb was inserted to form individual wells. This was removed once the stacking gel was set.

10ml 10% separating gel:

3.33ml 30% acrylamide *(National diagnostics, Hull, UK)*

3.75ml 1M Tris HCl pH 8.8

0.1ml 10% SDS *(Sigma)*

2.71ml dH_2O

0.1ml 10% ammonium persulphate *(Sigma)*

0.01ml TEMED *(Sigma)*

5ml 4% stacking gel:

0.65ml acrylamide *(National diagnostics)*

0.625ml 1M Tris HCl pH 6.8

0.05ml 10% SDS *(Sigma)*

3.62ml dH_2O

0.05ml 10% ammonium persulphate *(Sigma)*

0.01ml TEMED *(Sigma)*

Proteins were separated on the gel, across which a potential difference of 100V was applied, in a XL SureLock^{TM} tank *(Novex, San Diego, CA, USA)* containing Tris/Glycine/SDS running buffer *(Invitrogen)*. Following separation, the gel was removed from the cassette and proteins were transferred onto a Hybond-ECL nitrocellulose membrane *(Amersham)* using a horizontal semi-dry transfer method. 8 pieces of Whatman filter paper *(Whatman plc, Maidstone, Kent, U.K.)* saturated with transfer buffer were placed on the lower electrode of a Multiphor II *(Pharmacia LKB, Sweden)* horizontal semi-dry electroblot module. On top of these was placed the nitrocellulose membrane, then the gel, and above this was placed a further 8 pieces of

saturated Whatman filter paper. Transfer buffer consisted of Tris/Glycine/SDS running buffer with 5% methanol *(BDH)*. The top electrode was attached above the assembly of filter papers, membrane, and gel, and proteins were transferred using a current of 80mA (approximately 1mA per cm^2 of gel) for 2 hours. The quality of protein transfer was assessed by briefly staining the membrane with 2% Ponceau Red solution *(Sigma)*, followed by destaining by quick washing in double distilled water and then in PBS containing 0.1% Tween20 *(Sigma)*(PBST).

The membrane was blocked with PBST containing 5% dry non-fat milk for 120 minutes at RT, which was then discarded. Subsequently, 4ml of a 1:1000 dilution of goat anti-human/mouse CTGF antibody (L20: *Santa Cruz*) in PBST/5% milk was added and incubated using an orbital roller for 16 hours at 4°C. The membrane was washed three times with PBST, and then a horseradish peroxidase-conjugated rabbit anti-goat IgG *(Dako)* was added at a 1:2000 dilution in PBST/5% milk for 60 minutes followed by three washes in PBS-tween. Excess wash solution was drained off, and 2ml of ECL Plus *(Amersham)* was spread over the membrane and incubated for 1 minute. Excess reagent was drained, and the membrane was exposed in a dark room to autoradiography film *(Kodak)*, for between 30 secs and 10 minutes, which was then developed using an automated Kodak M35A X-Omat Processor *(Kodak)*.

Membranes were also stripped and reprobed with a rabbit anti-human actin antibody *(Santa Cruz)* at a 1:2000 dilution for 2 h at room temperature, followed a HRP-conjugated goat anti-rabbit secondary antibody (*Dako*, 1:2000 dilution) and enhanced chemiluminescence with ECL Plus *(Amersham)* as outlined above to allow assessment of equality of protein loading. Stripping was undertaken by immersing the membrane in a buffer containing:

100 mM 2-β-mercaptoethanol *(Sigma)*
2% SDS *(Sigma)*
62.5 mM Tris-Cl pH 6.7

The membrane was incubated at 50°C for 30 min in stripping buffer and then rinsed thoroughly with PBST.

2.3.6. CTGF protein detection by immunoflourescence

CTGF protein expression was also examined by immunocytofluorescence. $2x10^4$ A549 cells in 300µl F12K/ 10% FCS were seeded in each well of a 8 well Lab-tek chamber slide *(Nunc)*, and left to adhere to the slide in a humidified atmosphere with 5% CO2 at 37°C overnight. Medium was then removed, and replaced with serum-free medium for a further 24 hours. This medium was aspirated, and cells were then exposed to thrombin (25nM) in 200µl serum-free F12K for 4 hours. Cells were washed 3 times in PBS, and then fixed and permeabilised using ice-cold methanol for 45 seconds, before being washed again in PBS at 4°C.

Fixed cells were incubated with goat anti-human/mouse CTGF antibody (L20, *Santa Cruz*), at a dilution of 1:50 in 200µl PBS at room temperature (RT) for 60 minutes. Cells were washed 3 times in PBS and then non-specific binding sites were blocked by incubation for 5 minutes at RT with normal rabbit serum *(Dako)* at a dilution of 1:20 in 200µl PBS. After a further PBS wash, cells were incubated for 30 minutes with rabbit anti-goat-FITC conjugated antibody (1:50 in PBS)*(Dako)* at RT in the dark. This medium was aspirated, and the side-walls of the chambers were carefully removed. The slide was washed a further 3 times with PBS. Coverslips were mounted onto each slide using 3 drops of vectorshield mounting medium *(Vector Laboratories)* containing the 4'6-Dimidino-2-phenyindole (DAPI) high sensitivity nucleic acid stain. Images were captured using an inverted fluorescence microscope (*Zeiss* Axioskop 2) and photographed using an Olympus C-35DA-2 camera *(Olympus)*.

2.3.7. Mink lung epithelial cell bioassay for TGF-β

TGF-β in cell culture supernatants was measured using a mink lung epithelial cell bioassay. This assay was originally developed by Abe *et al* (Abe et al. 1994) and is based on the ability of TGF-β to specifically induce PAI-1 gene expression in mink lung epithelial cells stably transfected with an expression construct containing a truncated PAI-1 promoter fused to the firefly luciferase reporter gene. Exposure of these cells to TGF-β increases in luciferase activity in cell lysates in a dose-dependent manner. Importantly, the promoter fragment is only minimally influenced by other known inducers of PAI-1 expression such as bFGF, PDGF-BB, rIL-1α, and EGF.

Hence the assay is highly specific for TGF-β activity. In order to measure total TGF-β levels, the latent pool of TGF-β must first be activated by the addition of acid to samples, or heating them at 80°C for 10 minutes. The latter method was utilised for experiments outlined in this thesis because previous work in the laboratory had shown that acidification of samples interfered with the bioassay.

For all experiments, the assay was performed by plating stably transfected mink lung epithelial cells (as described above: MLEC) at a density of 1.6 x 10^4 cells/well in DMEM with 10% FCS in a 96-well tissue culture plate. After allowing cells to adhere for 3 hours the media was aspirated and cells were washed in serum-free medium. 100µl recombinant porcine TGF-β_1 (R&D Systems) standards (0.1-1µg/ml in the same culture medium as the samples) or heat-activated samples (diluted by between 4 and 6-fold so that TGF-β values were within the assay range) were added in triplicate to the plated MLEC. The plate was incubated for 14 hours at 37°C in a humidified atmosphere containing 5% CO_2. Media was then aspirated and the cells were washed in PBS, which was also then aspirated. Subsequently 100µl of luciferase reporter gene assay lysis buffer *(Roche)* was added to each well, and the plate agitated for 25 minutes. 20µl of each lysate was then transferred into the corresponding well of a 96-well opaque white optiplate *(Nunc).* The luciferase substrate *(Promega, Hampshire, UK),* was automatically added, and a luminescence reading obtained using the Tropix TR717 Microplate Luminometer *(Applied Biosystems).* Actual values for active TGF-β in samples were derived by comparison of luminescence readings against those of the standard curve generated in parallel using known concentrations of porcine TGF-β_1 .

2.4. Statistical analysis and expression of data

2.4.1. Statistical methods utilised

All data in figures are presented as mean ± standard error of the mean. Statistical comparison was performed between two treatment groups by Student's t test *(Microsoft Excel)*; and between multiple treatment groups by One-Way ANOVA with Student-Newman-Keuls (parametric data) or Dunn's test (non-parametric data) for post-hoc pair-wise multiple comparisons, using Sigmastat software *(SYSTAT Software Inc, Chicago, IL, USA)*. Assessment of CCL2 and TGF-β release in response to thrombin or Factor Xa over a time-course was by analysed by Two-Way ANOVA with Student-Newman-Keuls or Dunn's test for post-hoc pair-wise comparison (SigmaStat). A p value of less than 0.05 was considered significant.

2.4.2. Description of fold changes in pro-inflammatory and pro-fibrotic mediators in response to bleomycin in mice

When there were no apparent differences at baseline (saline-instilled) between the two mouse genotypes, fold changes in levels of mediators in response to bleomycin were expressed relative to a common baseline (i.e. the average of both saline-instilled WT and saline-instilled $PAR_1^{-/-}$ mice). Where there were significant differences at baseline, fold changes in response to bleomycin were expressed relative to levels in saline-treated mice of the same genotype.

2.4.3. Description of *in vitro* gene expression studies

mRNA levels in response to treatment were expressed relative to their respective time-point matched control. Relative mRNA levels (to control) in response to thrombin and TFLLR-NH_2 on A549 and BEAS-2B cells were combined into single graphs and the media controls replaced by a single dotted line for the sake of simplicity.

2.4.4. Description of the effect of inhibitors on mediator mRNA/ protein levels *in vitro*

As mentioned previously, the concentration of DMSO vehicle was standardised amongst all samples and controls. A series of concentrations of inhibitor were used in parallel. Fold increases of mediator levels in response to stimulus (e.g. thrombin) were calculated relative to their respective controls, which each contained the same amount of inhibitor and vehicle as stimulated samples. The percentage increase in response to stimulus without inhibitor was defined as 100%.

Percentage increase with inhibitor present is therefore given by the equation:

(fold increase above control with inhibitor / fold increase above control without inhibitor) x 100

The percentage inhibition is therefore: 100 - percentage increase.

3. RESULTS

3.1. The response to bleomycin-induced lung injury in the PAR_1-knockout ($PAR_1^{-/-}$) mouse

3.1.1. Introduction

As has been described previously, levels of coagulation proteinases are increased in fibroproliferative lung disorders (Chapman et al. 1986),(Ikeda et al. 1989), (Kotani et al. 1995),(Ohba et al. 1994),(Bachofen & Weibel 1982),(Dik et al. 2003),(Peyrol et al. 1990). Thrombin that is generated following extravascular activation of coagulation is able to exert potent pro-inflammatory and pro-fibrotic effects via the activation of its major cellular receptor PAR_1. Direct evidence supporting an important potential role for PAR_1 in injury responses is provided by studies showing that PAR_1 deficiency affords protection from experimentally-induced injuries to the kidney (Cunningham et al. 2000), synovium (Yang et al. 2005) and brain (Junge et al. 2003) in mice. In this thesis, the biological relevance of PAR_1 to injury within the lung was examined. Responses of wild-type (WT) and $PAR_1^{-/-}$ mice were compared following inflammatory and fibrotic responses initiated by the intra-tracheal administration of bleomycin. (These experiments were undertaken following basic preliminary data obtained with Dr David Howell suggesting PAR_1 deficiency conferred a reduction in lung collagen accumulation 14 days following bleomycin-induced lung injury. Those results are displayed in **Appendix A1**).

3.1.2. PAR_1 deficiency affords protection from bleomycin-induced lung inflammation

The early response to intra-tracheal administration of bleomycin in mice is characterized by a dramatic increase in microvascular leak and inflammatory cell recruitment (Thrall & Scalise 1995). The effect of PAR_1 deficiency on these processes was examined by assessing lung weight, BALF total cell number and BALF protein 6 days after bleomycin injury. BALF protein content was taken to represent vascular permeability and epithelial/endothelial barrier integrity. (Since lung weight, BALF

total cell number, and BALF protein were not significantly different in saline-instilled $PAR_1^{-/-}$ and saline-instilled WT mice, fold increases in response to bleomycin for each of these parameters were calculated relative to the average of all the saline-instilled mice of both genotypes).

Lung weight was first examined to assess the extent that it increased in response to bleomycin in WT and $PAR_1^{-/-}$ mice. The basis for this increase in lung weight is likely to be cellular infiltration, and congestion with protein and fluid. The increase in lung weight 6 days following bleomycin (**Figure 3.1.1, Panel A**), in lungs that had been lavaged, was 41.6 ± 2.9% in WT ($p<0.01$), but only 18.9 ± 3.6% in $PAR_1^{-/-}$ mice ($p<0.01$). (A similar attenuation in lung weight in $PAR_1^{-/-}$ mice 6 days following bleomycin was obtained in a separate similar experiment involving non-lavaged lungs shown in **Appendix A2**).

There was no significant difference in BALF total leukocyte numbers between both genotypes given intratracheal saline (**Figure 3.1.1, Panel B**). BALF total leucocyte numbers increased by 104.7 ± 17.8% in WT ($p<0.05$), and there was a non-significant trend for a 29.8 ± 22.7% increase in $PAR_1^{-/-}$ mice. This represents an attenuation of the characteristic bleomycin-associated increase in BALF cell number in $PAR_1^{-/-}$ mice. The relative proportions of leucocyte subsets within BALF were not further examined in this thesis, although in previous work by Dr David Howell, they were found to be similar in $PAR_1^{-/-}$ and WT mice following bleomycin.

There was no difference between BALF total protein levels in WT and $PAR_1^{-/-}$ mice given intratracheal saline (**Figure 3.1.1, Panel C**). Following instillation of bleomycin, BALF protein increased by 11.4 ± 0.7 fold in WT ($p<0.05$), but only by 6.0 ± 1.1 fold in $PAR_1^{-/-}$ mice ($p<0.05$). This represents an attenuation of the characteristic bleomycin-associated increase in BALF total protein in $PAR_1^{-/-}$ mice.

Figure 3.1.1.

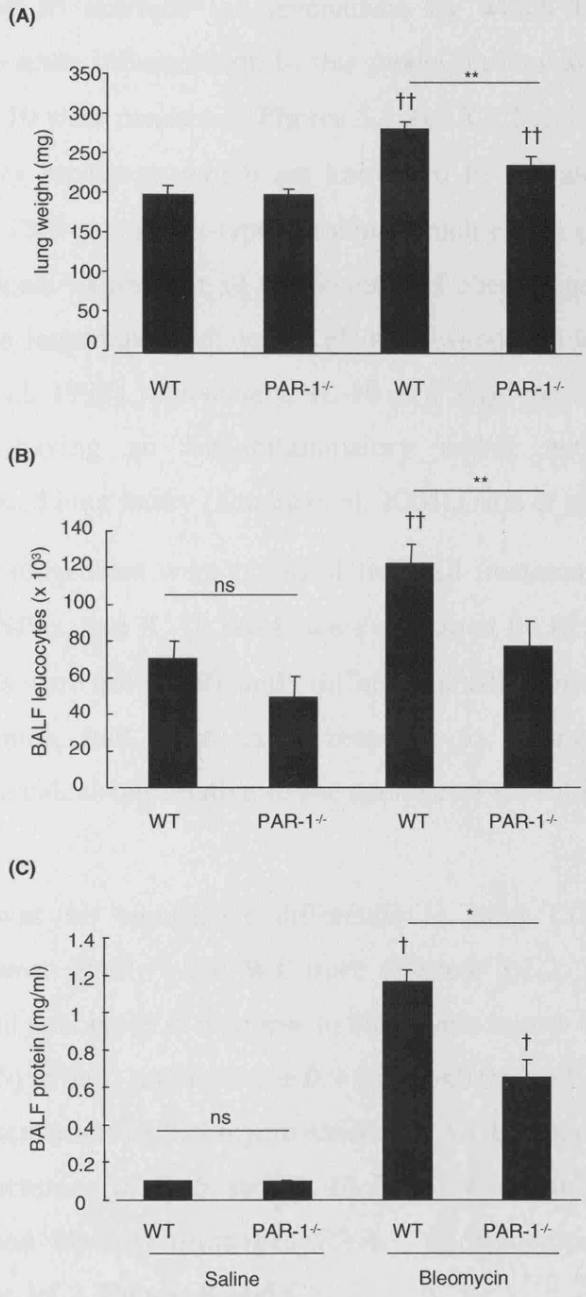


Figure 3.1.1: Increases in lung weight, BALF total inflammatory cell number and BALF total protein concentration in response to bleomycin are attenuated in $PAR_1^{-/-}$ mice.

(**A**) Total weight of excised lungs, (**B**) total number of inflammatory cells in BALF, and (**C**) total protein concentration in BALF obtained 6 days after intratracheal instillation of saline or bleomycin. Data represent the mean ± SEM of values obtained in 9-11 mice per group. **p<0.01; *p<0.05 difference between bleomycin-treated $PAR_1^{-/-}$ and bleomycin-treated WT mice. ns: no significant difference between genotype. ††p<0.01; †p<0.05 compared with saline-treated mice (One Way ANOVA with Student-Newman-Keuls (**A & B**) and Dunn's (**C**) post-hoc tests).

3.1.3. Potential PAR_1-dependent mediators involved in bleomycin-induced lung inflammation.

To begin to examine the mechanism by which PAR_1-deficiency affords protection from acute inflammation in this model, pulmonary levels of CCL2, IL-6, TNF-α, and IL-10 were measured (**Figure 3.1.2**). CCL2 and IL-6 are PAR_1 inducible pro-inflammatory mediators which are known to be elevated following bleomycin administration. TNF-α is a Th_1-type cytokine which exerts pro-inflammatory effects via triggering local expression of cytokines and chemokines, and which has been implicated as an important mediator of bleomycin-induced lung injury (Piguet et al. 1989),(Ortiz et al. 1998). Conversely, IL-10 is a Th_2-type cytokine which has been implicated as having an anti-inflammatory and/or anti-fibrotic influence on bleomycin-induced lung injury (Kradin et al. 2004),(Arai et al. 2000).

Lung homogenates were prepared from all treatment groups at 6 days, and CCL2, IL-6, TNF-α, and IL-10 levels were measured by ELISA. Since CCL2, IL-6, and IL-10 levels were not significantly different in saline-instilled $PAR_1^{-/-}$ and saline-instilled WT mice, fold increases in response to bleomycin for each of these parameters were calculated relative to the average of *all* saline-instilled mice of both genotypes.

There was no significant difference in lung CCL2 levels after saline instillation between $PAR_1^{-/-}$ and WT mice (**Figure 3.1.2, Panel A**). CCL2 levels increased in both genotypes in response to bleomycin injury. CCL2 increased by 6.5 ± 0.4 fold ($p<0.05$) in WT; and by 4.2 ± 0.9 fold ($p<0.05$) in $PAR_1^{-/-}$ mice, representing a significant attenuation in bleomycin-associated CCL2 increase in $PAR_1^{-/-}$ mice. In contrast, the increases in IL-6 and IL-10 levels were similar in both bleomycin-instilled WT and bleomycin-instilled $PAR_1^{-/-}$ mice, compared with saline-treated controls (**Figure 3.1.2, Panels B and C**).

Interestingly, there was significantly more TNF-α in saline-instilled $PAR_1^{-/-}$ than saline-instilled WT mice ($p<0.05$). (**Figure 3.1.2, Panel D**). In this case, because there was a difference in TNF-α levels in saline-instilled mice of each genotype, the fold increases in TNF-α were calculated relative to the average of the saline-instilled group of *the same genotype*, and not relative to the average of *all* saline-instilled mice of both genotypes (as with the other fold increases described in these results). In

response to bleomycin, the increase in TNF-α in WT mice (WT saline to WT bleomycin) was 2.0 ± 0.1 fold ($p<0.05$) but was only 1.2 ± 0.1 fold (ns) in $PAR_1^{-/-}$ mice ($PAR_1^{-/-}$ saline to $PAR_1^{-/-}$ bleomycin).

3.1.4. Comparison of early injury and local fibrin deposition following bleomycin in $PAR_1^{-/-}$ and WT mice

BALF lactate dehydrogenase (LDH) is a useful indicator of lung damage (reviewed in (Drent et al. 1996)) since cell damage or death results in release of LDH into the extracellular space. To examine whether attenuated responses in $PAR_1^{-/-}$ mice may result from a difference in the immediate injury caused by bleomycin compared to that in WT mice, BALF LDH activity was measured 24 hours following bleomycin or saline. LDH activity was increased by a similar amount in each genotype (**Figure 3.1.3, panel A**), suggesting that attenuated responses in $PAR_1^{-/-}$ mice are unlikely to be due to differences in the initial lung injury caused by bleomycin.

Fibrin has been shown to exert a pro-fibrotic influence *in vitro* (Gray et al. 1995). It accumulates in the alveolar space in response to bleomycin in mice (Olman et al. 1996), and is a prominent histological feature of ARDS. In order to examine whether fibrin generation might differ between the two genotypes, the extent of fibrin deposition was examined in $PAR_1^{-/-}$ and WT mice 7 days following bleomcyin or saline instillation: a time point around which procoagulant activity has been shown to peak in the bleomycin model. (Tani et al. 1991). Very few fibrin fibres were found in saline-instilled animals (**Figure 3.1.3, Panels B & C**). After bleomycin, fibrin fibres, visible as fine red strands, were essentially only seen in inflammatory foci associated with bleomycin injury. The extent of fibrin deposition within these inflammatory foci appeared equivalent for $PAR_1^{-/-}$ and WT mice (**Figure 3.1.3, Panels D & E**). These findings suggest that attenuated responses in $PAR_1^{-/-}$ mice are independent of fibrin formation.

Figure 3.1.2.

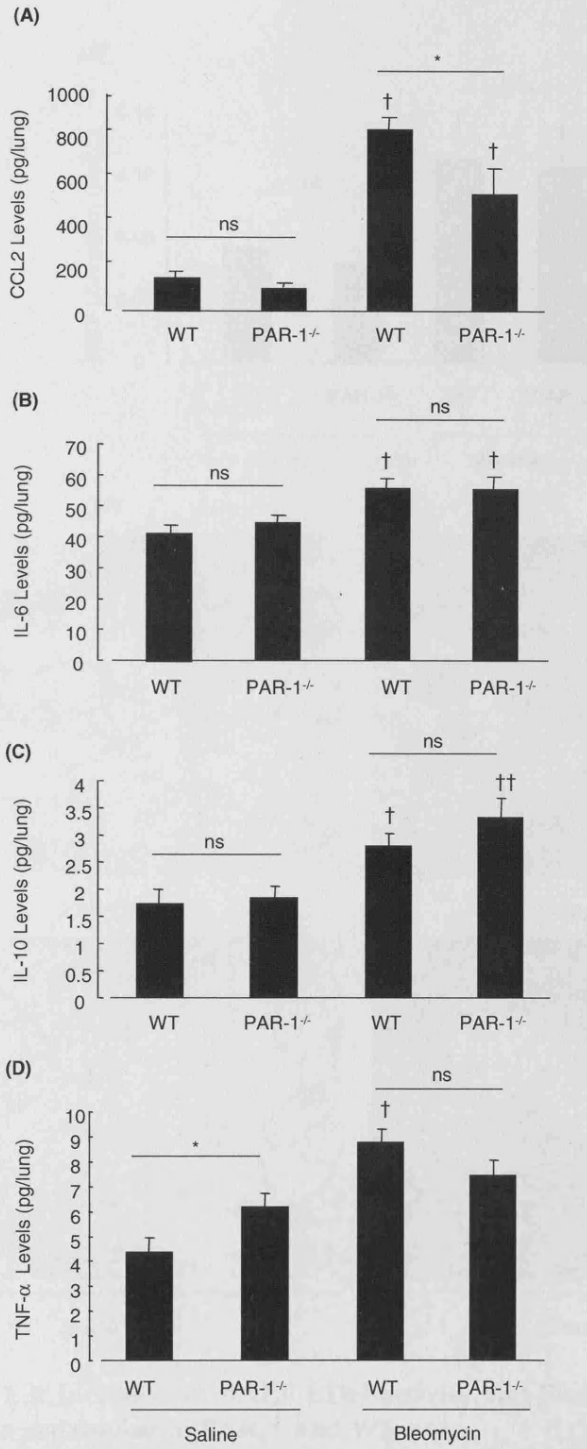


Figure 3.1.2: $PAR_1^{-/-}$ mice have reduced lung levels of CCL2 but not IL-6, IL-10, or TNF-α following intratracheal bleomycin.

Figure shows levels of CCL2 (**A**), IL-6 (**B**), IL-10 (**C**), and TNF-α (**D**) protein, as measured by ELISA, in lung homogenates 6 days after intratracheal saline or bleomycin. Data represent the mean ± SEM of values obtained in 8-11 mice per group. *$p<0.05$ difference between $PAR_1^{-/-}$ and WT mice receiving the same treatment. ns: no significant difference between genotype. †$p<0.05$ compared with saline-treated mice (One Way ANOVA with Student-Newman-Keuls post-hoc tests).

Figure 3.1.3:

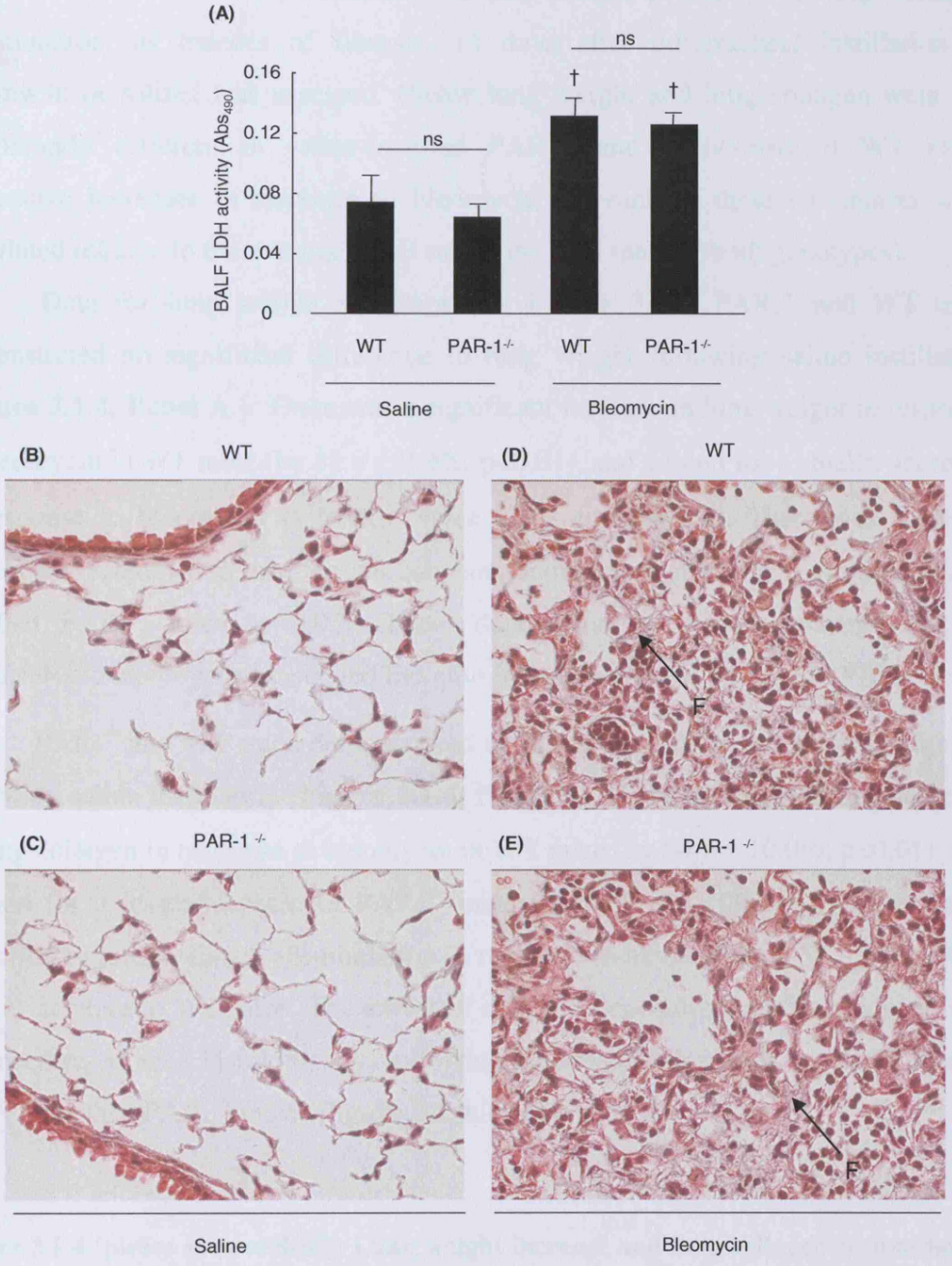


Figure 3.1.3: Increases in BALF LDH activity and fibrin deposition following bleomycin are similar in $PAR_1^{-/-}$ and WT mice.

A. LDH activity in BALF obtained 24 hours after intratracheal instillation of saline or bleomycin. Data represent the mean ± SEM of 5 mice per group. † $p<0.05$ comparison with saline treated mice (One Way ANOVA with Student-Newman Keuls post-hoc tests). (**B-E**): Representative images (x 400 original magnification) of fibrin staining in lung sections from mice treated 7 days previously with saline (**B & C**) or bleomycin (**D & E**). (F = fibrin fibres).

3.1.5. PAR_1 deficiency affords protection from bleomycin-induced lung fibrosis

The effect of PAR_1 deficiency on lung weight increase, and lung collagen accumulation, as indeces of fibrosis, 14 days after intratracheal instillation of bleomycin or saline, was assessed. (Since lung weight and lung collagen were not significantly different in saline-instilled $PAR_1^{-/-}$ and saline-instilled WT mice, percentage increases in response to bleomycin for each of these parameters were calculated relative to the average of *all* saline-instilled mice of both genotypes).

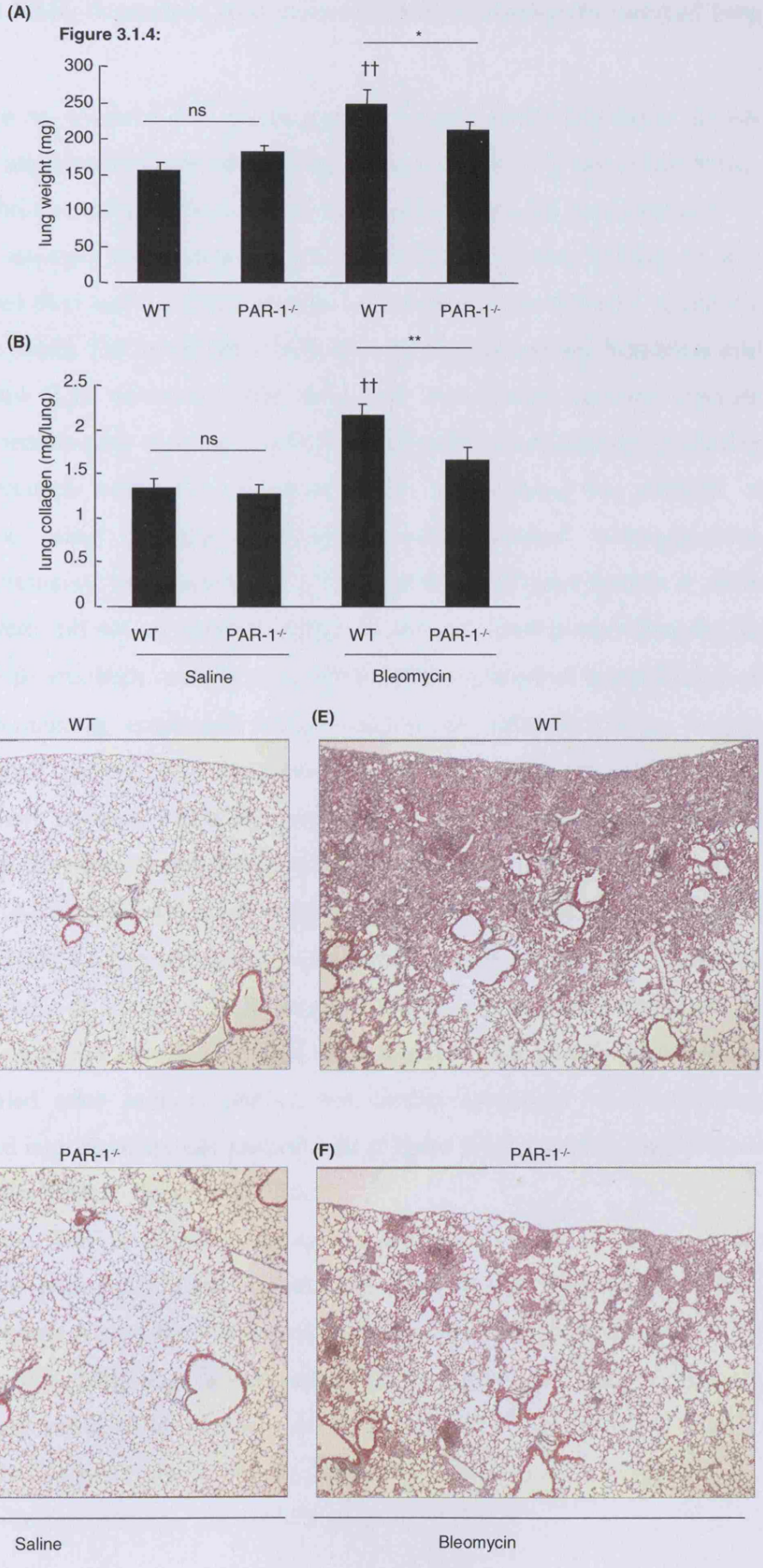
Data for lung weight are shown in **Figure 3.1.4**. $PAR_1^{-/-}$ and WT mice demonstrated no significant difference in lung weight following saline instillation (**Figure 3.1.4, Panel A.**). There was a significant increase in lung weight in response to bleomycin in WT mice (by 52.8 ± 9.9%; $p<0.01$), and a trend for a smaller increase in response to bleomycin in $PAR_1^{-/-}$ mice (29.4 ± 7.0%; ns). There was a also a significant difference in lung weight between bleomycin-instilled WT and bleomycin-instilled $PAR_1^{-/-}$ mice ($p<0.05$). These data demonstrate an attenuation in the characteristic bleomycin-associated increase in lung weight at 14 days in $PAR_1^{-/-}$ mice.

$PAR_1^{-/-}$ and WT mice demonstrated no significant difference in lung collagen following saline instillation (**Figure 3.1.4, Panel B**). There was a significant increase in lung collagen in response to bleomycin in WT mice (by 64.2 ± 10.0%; $p<0.01$), and a trend for a lesser increase in $PAR_1^{-/-}$ mice (by 26.8 ± 11.0%; ns). There was a reduction in lung collagen accumulation in response to bleomycin by 58.2 ± 17.1% in $PAR_1^{-/-}$ relative to WT mice. The extent of collagen deposition and distortion of lung architecture, as seen histologically, following bleomycin injury was markedly greater for WT than for $PAR_1^{-/-}$ mice (**Figure 3.1.4, Panels C to F**).

Figure 3.1.4 (please see overleaf): Lung weight increase and lung collagen accumulation is attenuated in $PAR_1^{-/-}$ mice at 14 days.

(**A**) Total lung weight of excised lungs from mice 14 days after saline and bleomycin instillations. (**B**) Total lung collagen (in these same animals), as measured by reverse-phase HPLC quantitation of lung hydroxyproline in acid hydrolysates of pulverized lung. Data represent the mean ± SEM of values obtained in 9-11 mice per group. **$p<0.01$, *$p<0.05$ difference between bleomycin-treated $PAR_1^{-/-}$ and bleomycin-treated WT mice. ns: no significant difference between genotype. ††$p<0.01$ compared with saline-treated mice (One Way ANOVA with Student-Newman-Keuls post-hoc tests). (**C-F**): Representative images (x 40 original magnification) of mice treated previously with saline (**C & D**) or bleomycin (**E & F**).

Figure 3.1.4:


(A)
lung weight (mg)
300
250
200
150
100
50
0
ns
*
††
WT
PAR-1-/-
WT
PAR-1-/-
(B)
lung collagen (mg/lung)
2.5
2
1.5
1
0.5
0
ns
**
††
WT
PAR-1-/-
WT
PAR-1-/-
Saline
Bleomycin
(C)
WT
(E)
WT
(D)
PAR-1-/-
(F)
PAR-1-/-
Saline
Bleomycin

3.1.6. Potential PAR_1-dependent mediators involved in bleomycin-induced lung fibrosis.

To begin to examine the mechanism by which PAR_1-deficiency affords protection from experimental lung fibrosis, the pulmonary levels of two potent PAR_1-inducible pro-fibrotic mediators known to be elevated in this model were assessed.

Initially attempts were made to identify CTGF by western blotting of lung homogenates, and then assess relative protein expression by densitometry of specific immunoreactive bands. The bands obtained by this method (**please see Materials and Methods, Figure 2.1**), however, were faint and inconsistent between separate experiments. Attempts were therefore made to optimise protein extraction by altering the salt concentration within the extraction buffer, and altering the methods of disrupting tissue using differing freeze-thaw and mechanical homogenisation protocols. Unfortunately, immunoreactive CTGF bands of sufficient quality to allow quantification were still not reproducibly obtained. Instead, since immunostaining for CTGF and TGF-β_1 was both specific and reproducible, a method of quantification of positive immunostaining, employing image analysis of light microscopy images captured by digital camera, was therefore devised as a means of assessment of expression of these proteins within the lung (**please see Materials and Methods: 2.2.5.1 & 2.2.5.2 for more detail of this methodology**).

Figures 3.1.5 and 3.1.6 show representative immunohistochemical staining for CTGF and TGFβ_1 following intratracheal instillation of bleomycin or saline in WT and $PAR_1^{-/-}$ mice at 14 days. CTGF immunostaining was predominantly localised to bronchiolar epithelium in saline-instilled mice (**Figure 3.1.5, panels A and B**). In bleomycin-instilled mice immunostaining was further associated with infiltrating macrophages and interstitial spindle-shaped cells (**Figure 3.1.5, panels C and D**), but was less intense for $PAR_1^{-/-}$ than for WT mice.

In contrast, TGF-β_1 immunostaining was predominantly localised to alveolar macrophages, and both bronchiolar and alveolar epithelium in saline-instilled mice (**Figure 3.1.6, panels A and B**). In bleomycin-instilled mice, the intensity of staining increased markedly and was associated with the alveolar wall, infiltrating macrophages, and extracellular matrix associated with fibrotic foci (**Figure 3.1.6, panels C and D**). Staining intensity was again attenuated in bleomycin-injured $PAR_1^{-/-}$ compared with correspondingly injured WT mice.

Figure 3.1.5:

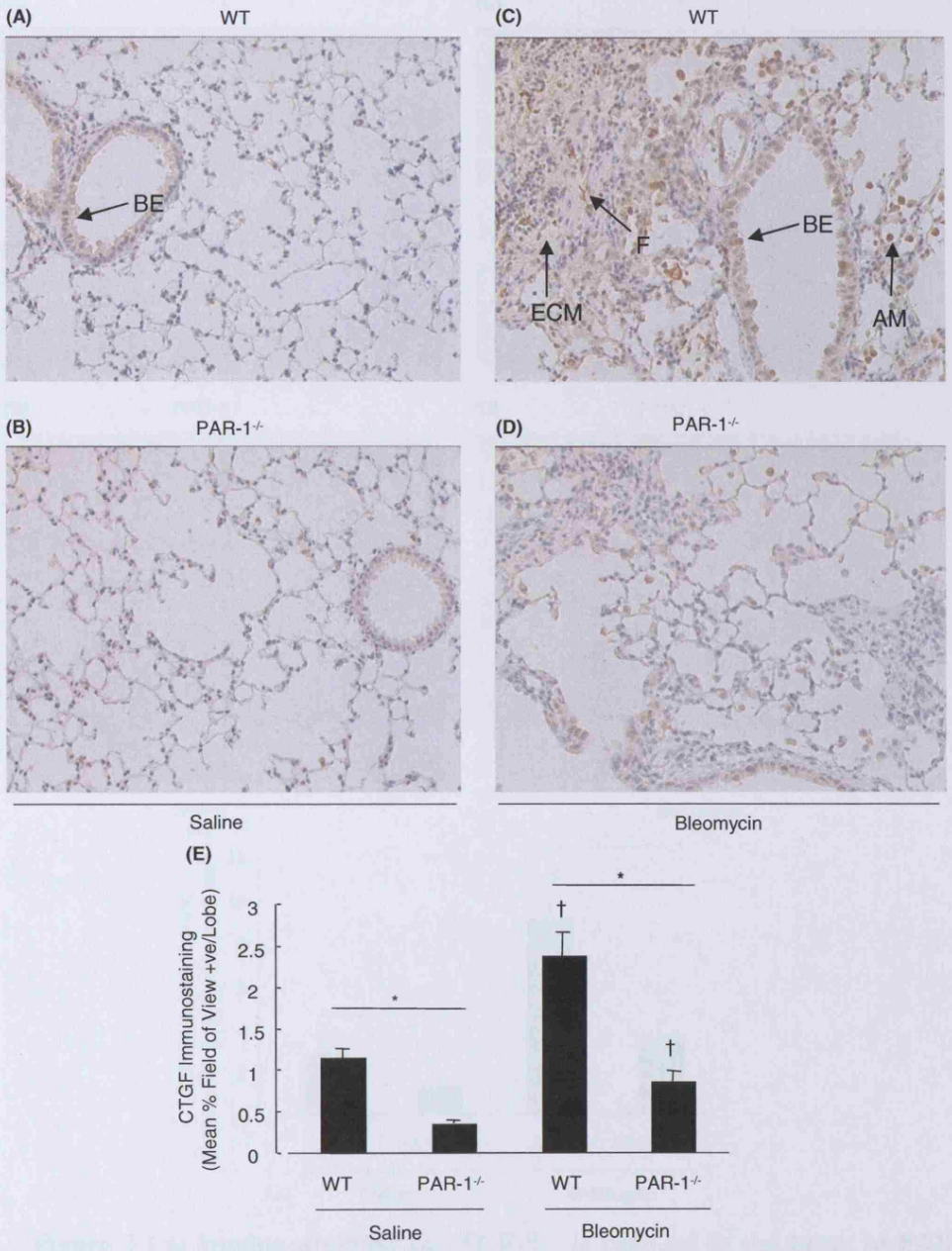


Figure 3.1.5: Immunostaining for CTGF is reduced in the lungs of $PAR_1^{-/-}$ mice. **(A-D)** Immunostaining for CTGF on representative lung tissue sections from $PAR_1^{-/-}$ and WT mice 14 days after saline and bleomycin instillations, original magnification x 200. **(A & B)** In saline-instilled mice, positive immunostaining (brown) was predominantly localised to bronchiolar epithelium (BE). **(C & D)** In bleomycin-instilled mice, immunostaining was also associated with alveolar macrophages (AM), interstitial spindle-shaped cells (F), and adjacent extracellular matrix (ECM), but was less intense in $PAR_1^{-/-}$ mice. **(E)** Quantitation of immunostaining (semi-quantitative immunohistochemical image-analysis). Data represent mean ± SEM of values obtained in at least 4 mice per group. *$p<0.05$ difference between $PAR_1^{-/-}$ and WT mice following instillation of saline or bleomycin. † $p<0.05$ difference in bleomycin-instilled compared with saline-instilled mice of the same genotype (One Way ANOVA with Dunn's post hoc tests).

Figure 3.1.6:

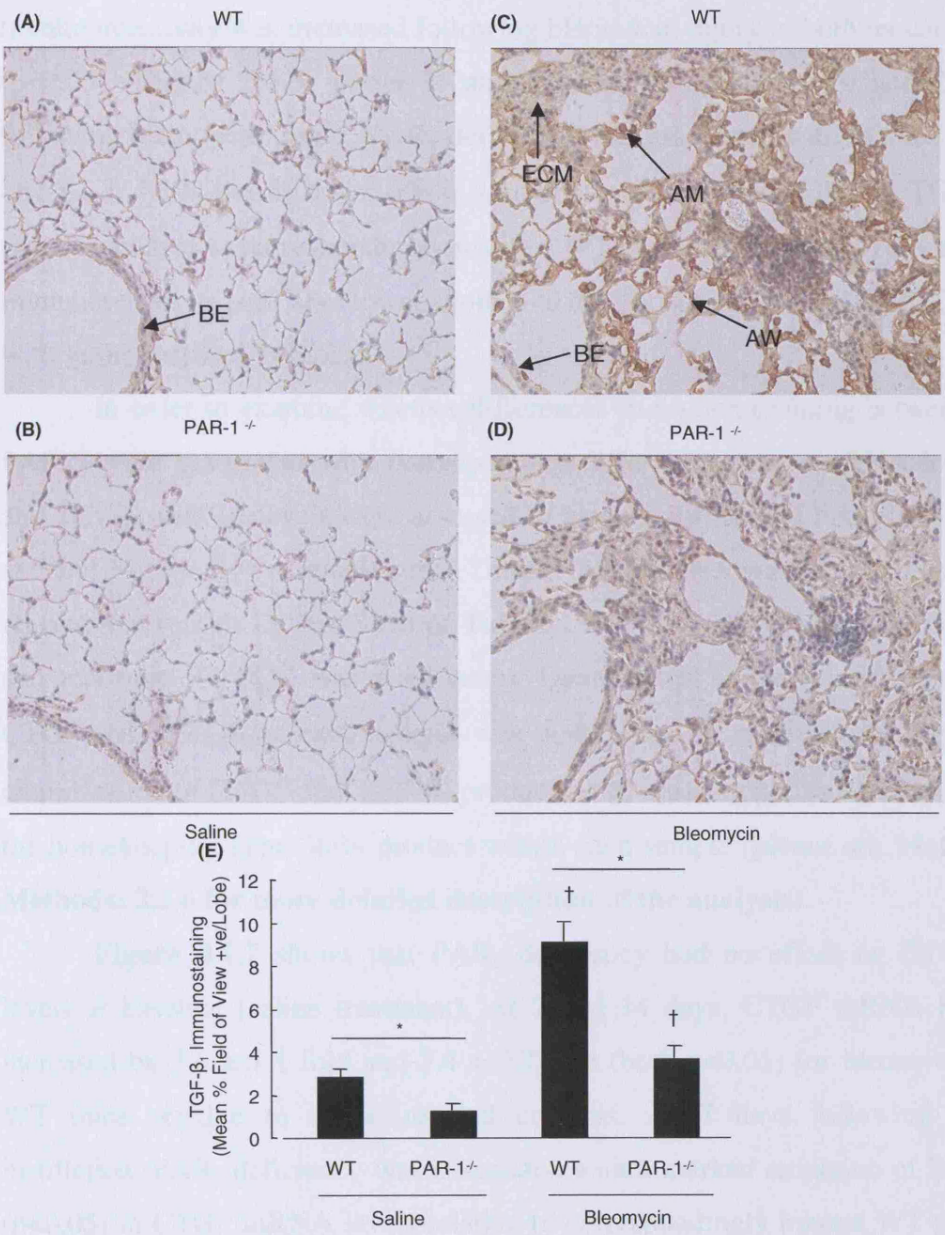


Figure 3.1.6: Immunostaining for TGF-β_1 is reduced in the lungs of PAR$_1^{-/-}$ mice. (A-D) Immunostaining for TGF-β_1 on representative lung tissue sections from PAR$_1^{-/-}$ and WT mice 14 days after saline and bleomycin instillations, original magnification x 200. **(A & B)** In saline-treated mice, positive immunostaining (brown) was weak and localised to bronchiolar epithelium (BE), alveolar macrophages (AM), and alveolar epithelium. **(C & D)** In bleomycin-treated mice, immunostaining increased markedly on the alveolar wall (AW), infiltrating macrophages (AM), and extracellular matrix associated with fibrotic foci (ECM), but was less intense in PAR$_1^{-/-}$ mice. **(E)** Quantitation of immunostaining (semi-quantitative immunohistochemical image analysis). Data represent mean ± SEM of values obtained in at least 4 mice per group. *$p<0.05$ difference between PAR$_1^{-/-}$ and WT mice following instillation of saline or bleomycin. †$p<0.05$ difference in bleomycin-instilled compared with saline-instilled mice of the same genotype (One Way ANOVA with Dunn's post-hoc tests).

Semi-quantitative image-analysis revealed that CTGF and TGF-β_1 immunoreactivity was increased following bleomycin injury in both mouse genotypes ($p<0.05$) **(Figure 3.1.5, panel E and Figure 3.1.6, panel E)**, and further that following bleomycin injury, PAR_1 deficiency was associated with marked reductions of 63.1 ± 4.2% and 60.4 ± 6.0% in immunoreactivity for CTGF and TGF-β_1 (both $p<0.05$) relative to correspondingly instilled WT mice. Of interest, CTGF and TGF-β_1 immunoreactivity was also lower (both $p<0.05$) for saline-instilled $PAR_1^{-/-}$ compared with saline-instilled WT mice.

In order to examine whether differences in immunostaining between WT and $PAR_1^{-/-}$ were associated with corresponding differences at the mRNA level, CTGF and TGF-β_1 mRNA levels were assessed in bleomycin-instilled $PAR_1^{-/-}$ and WT mice at 7 and 14 days by quantitative real-Time RT-PCR. RNA was extracted using TRIzol reagent and sent to Dr Bin Shan of Tulane University, USA, where cDNA synthesis and real-time RT-PCR was undertaken. Quantitation of the relative abundance of CTGF and TGF-β_1 in each sample was undertaken by relating crossing points for amplification of CTGF and TGF-β_1 products with crossing points for amplification of the housekeeping gene 36B4 product within each sample (**please see Materials and Methods: 2.2.6 for more detailed description of the analysis**).

Figure 3.1.7 shows that PAR_1 deficiency had no effect on CTGF mRNA levels at baseline (saline treatment). At 7 and 14 days, CTGF mRNA levels were increased by 3.6 ± 1.1 fold and 3.4 ± 0.7-fold (both $p<0.05$) for bleomycin-instilled WT mice relative to saline-instilled controls. At 7 days following bleomycin instillation, PAR_1 deficiency was associated with a marked reduction of 78.8 ± 5.0% ($p<0.05$) in CTGF mRNA levels relative to correspondingly injured WT mice. At 14 days, there was a trend for a reduction in CTGF mRNA levels of 43.7 ± 11.4% in bleomycin-instilled $PAR_1^{-/-}$ relative to bleomycin-instilled WT mice, but this failed to reach statistical significance.

Figure 3.1.8 shows that TGF-β_1 mRNA levels were similar in the two genotypes at baseline. At both 7 and 14 days, there was a trend towards an increase in TGF-β_1 mRNA levels in response to bleomycin injury in WT but not in $PAR_1^{-/-}$ mice relative to saline-instilled controls, although this failed to reach statistical significance (**Figure 3.1.8**).

Figure 3.1.7:

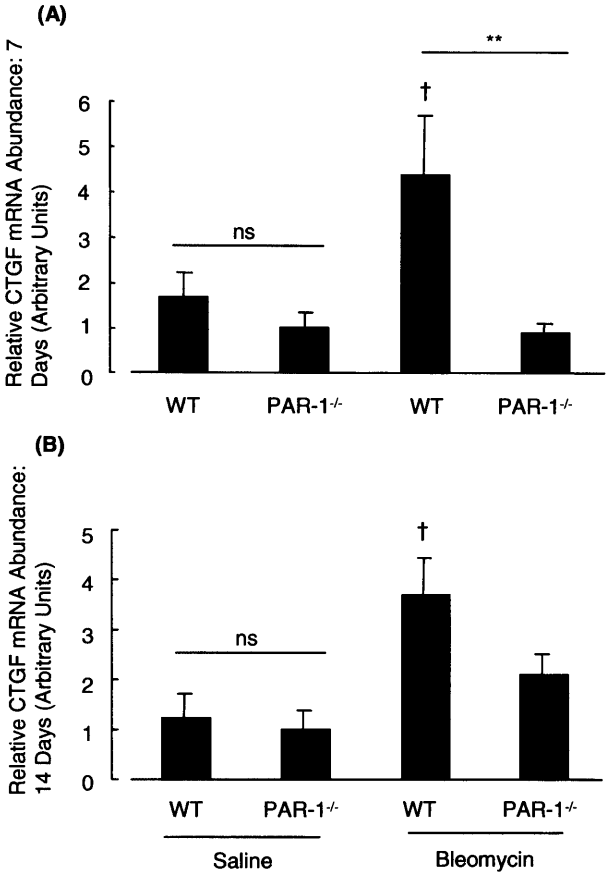


Figure 3.1.7: Pulmonary CTGF mRNA levels induced in response to bleomycin are attenuated in $PAR_1^{-/-}$ mice.

Relative abundance of CTGF mRNA, measured by real Time RT-PCR, is normalized for the internal control gene 36B4, and expressed in arbitrary units. **(A)** Relative abundance of CTGF mRNA in the lung 7 days after intratracheal saline or bleomycin. **(B)** Relative abundance of CTGF mRNA in the lung 14 days after intratracheal saline or bleomycin. Data represent the mean ± SEM of values obtained in 8 mice per group. $^{**}p<0.01$ difference between bleomycin-treated $PAR_1^{-/-}$ and bleomycin-treated WT mice. ns: no significant difference between genotype. †$p<0.05$ compared with saline-treated mice (One Way ANOVA with Student-Newman-Keuls post-hoc tests).

Figure 3.1.8:

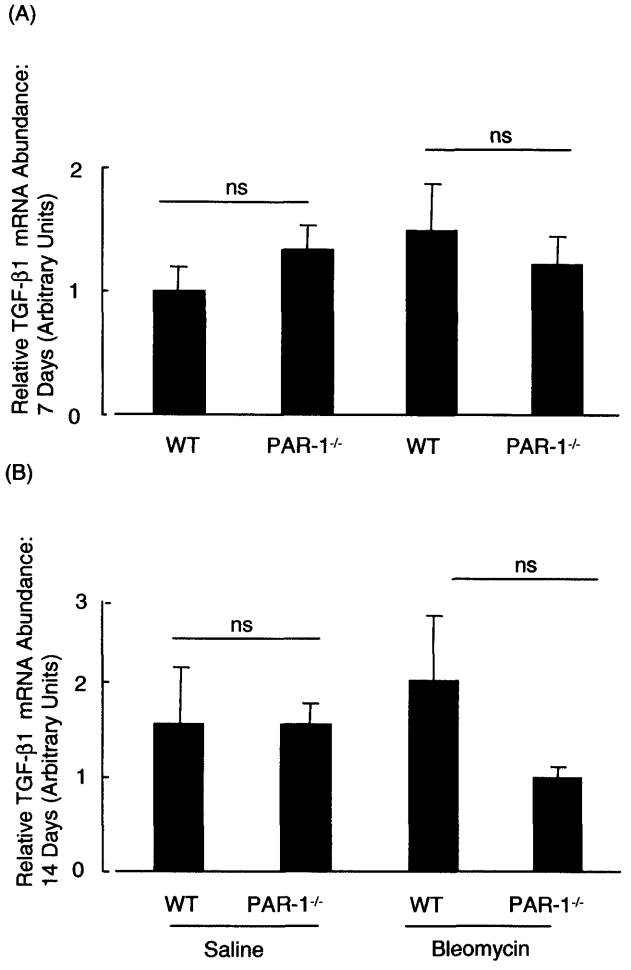


Figure 3.1.8: Pulmonary TGF-β_1 mRNA levels are unchanged in response to bleomycin in both WT and $PAR_1^{-/-}$ mice.

Relative abundance of TGF-β_1 mRNA, measured by Real Time RT-PCR, is normalized for the internal control gene 36B4, and expressed in arbitrary units. **(A)** Relative abundance of TGF-β_1 mRNA in the lung 7 days after intratracheal saline or bleomycin. **(B)** Relative abundance of TGF-β_1 mRNA in the lung 14 days after intratracheal saline or bleomycin. Data represent the mean ± SEM of values obtained in 8 mice per group. ns: no significant difference between genotype. ns: not significant (One Way ANOVA)

3.1.7. Summary

- PAR_1 deficiency is associated with an attenuation of the inflammatory response following bleomycin, as evidenced by diminution of bleomycin-induced increases in lung weight and BALF protein levels and leucocyte numbers at day 6.
- The attenuated inflammatory response in $PAR_1^{-/-}$ mice is associated with reduced lung levels of CCL2, but not IL-6 or IL-10.
- BALF LDH increased to an equivalent extent 24 hours after bleomycin instillation in WT and $PAR_1^{-/-}$ mice, suggesting that the initial injury to bleomycin is similar in both genotypes.
- Fibrin deposition in inflammatory foci was similar in WT and $PAR_1^{-/-}$ mice 7 days after bleomycin, suggesting that attenuated responses in $PAR_1^{-/-}$ mice are independent of fibrin formation.
- PAR_1 deficiency is associated with an attenuation of the fibrotic response following bleomycin, as evidenced by diminution of bleomycin-induced increases in lung weight and lung collagen at day 14.
- The attenuated fibrotic response in $PAR_1^{-/-}$ mice is associated with reduced lung CTGF and TGF-β_1 protein levels and CTGF mRNA levels.
- There were no significant increases in TGF-β_1 mRNA levels following bleomycin in either genotype.

3.2 The effect of PAR_1 activation on lung epithelial cell CCL2 and CTGF expression

In **Chapters 3.2** and **3.3**, some of the data presented pertains to the effect of PAR_1 activation on CCL2, CTGF, and TGF-β_1 mRNA levels in lung epithelial cells. It should be noted that in these experiments, RNA extracted from the same set of epithelial cells, exposed to various PAR_1 agonists and/or inhibitors, was utilized in separate real-time RT-PCR reactions by using separate specific PCR primer pairs for CCL2, CTGF, and TGF-β_1.

3.2A Effect of PAR_1 activation on lung epithelial CCL2 expression

3.2A.1. Introduction

Hyperplastic epithelial cells represent a potential source of pro-fibrotic mediators in fibroproliferative lung disease (Selman & Pardo 2006),(Khalil et al. 1991) and in the bleomycin model (Azuma et al. 2005a). Since protection from bleomycin-induced lung injury and fibrosis in $PAR_1^{-/-}$ mice is associated with reduced lung levels of the PAR_1-dependent mediators CCL2, CTGF, and TGF-β_1 (**Chapter 3.1**), it follows that each of these mediators might be involved in promoting inflammation and/or fibrosis following PAR_1 activation in the setting of lung injury. Therefore the influence of PAR_1 activation on the expression and release of these mediators by lung epithelial cells was investigated.

CCL2 may play an important role in inducing both inflammation and fibrosis following bleomycin injury. CCL2 is a potent chemoattractant for inflammatory cells, but can also promote fibrosis by upregulating TGF-β_1 and procollagen gene expression in fibroblasts (Gharaee-Kermani et al. 1996), downregulating epithelial cell production of the anti-fibrotic PGE_2 (Moore et al. 2003), and recruiting collagen-synthesising fibrocytes (Moore et al. 2005). Levels of CCL2 in BALF (Goodman et al. 1996) and serum (Suga et al. 1999) correlate with extent of lung injury and disease progression in ARDS and IPF. These observations implicate CCL2 in the pathogenesis of fibroproliferative lung disorders.

3.2A.2. Immunolocalisation of PAR_1 in mouse lung following bleomycin injury

PAR_1 is known to be widely expressed by a number of cell types present within the lung. PAR_1 immunostaining has previously been shown to increase following bleomycin injury in the rat lung (Howell et al. 2001). To investigate whether PAR_1 was similarly expressed in mouse lung, and to determine on which cells PAR_1 was most obviously immunolocalized to, immunostaining for PAR_1 was

performed on lung sections from WT mice instilled with either saline or bleomycin at 7 and 14 days (**Figure 3.2A.1 and 3.2A.2**). PAR_1 was predominantly localized to bronchiolar epithelial cells, alveolar macrophages, and endothelial cells in saline-instilled control mice (**Figure 3.2A.1, Panels A & B**). Following bleomycin injury, the intensity of immunostaining per cell was most obviously increased on type II alveolar epithelial and bronchial epithelial cells (**Figure 3.2A.1, Panels C & D; and Figure 3.2A.2, Panels C & D**), but also on alveolar macrophages (**Figure 3.2A.1, Panel C**) and spindle-shaped cells within the fibrotic matrix (**Figure 3.2A.1, Panel D**).

To further evaluate whether bleomycin-induced lung injury might influence local PAR_1 gene expression, PAR_1 mRNA levels following bleomycin were additionally assessed by real-time RT-PCR in cells recovered from BALF (predominantly macrophages) 14 days after bleomycin or saline administration. Lavaged cells were pooled from 10 mice instilled with intra-tracheal saline, and from 3 mice instilled with intra-tracheal bleomycin. Pools of cells from several mice were necessary because of the low number of cells and corresponding low yield of extractable RNA from bronchoalveolar lavage of a single mouse. Cell numbers in lavages of saline-instilled mice were much lower than in bleomycin-instilled mice. This necessitated pooling of lavages from a greater number of saline-instilled than bleomycin-instilled mice. Pooling of lavaged cells should also have made mRNA levels more representative of the group of mice from which cells were pooled rather than of individual mice. Relative abundance of PAR_1 mRNA was established following normalization of the abundance of the PAR_1 product to that of the 18S housekeeping gene product within each pooled sample (**please see Materials and Methods: 2.3.3.7 for more detailed description of this analysis**). PAR_1 mRNA levels were 5.3 times more abundant in bleomycin-treated compared to saline-treated mice (**Figure 3.2A.1, Panel E**). Taken together, these data support the notion that lung injury increases local expression of PAR_1.

Figure 3.2A.1:

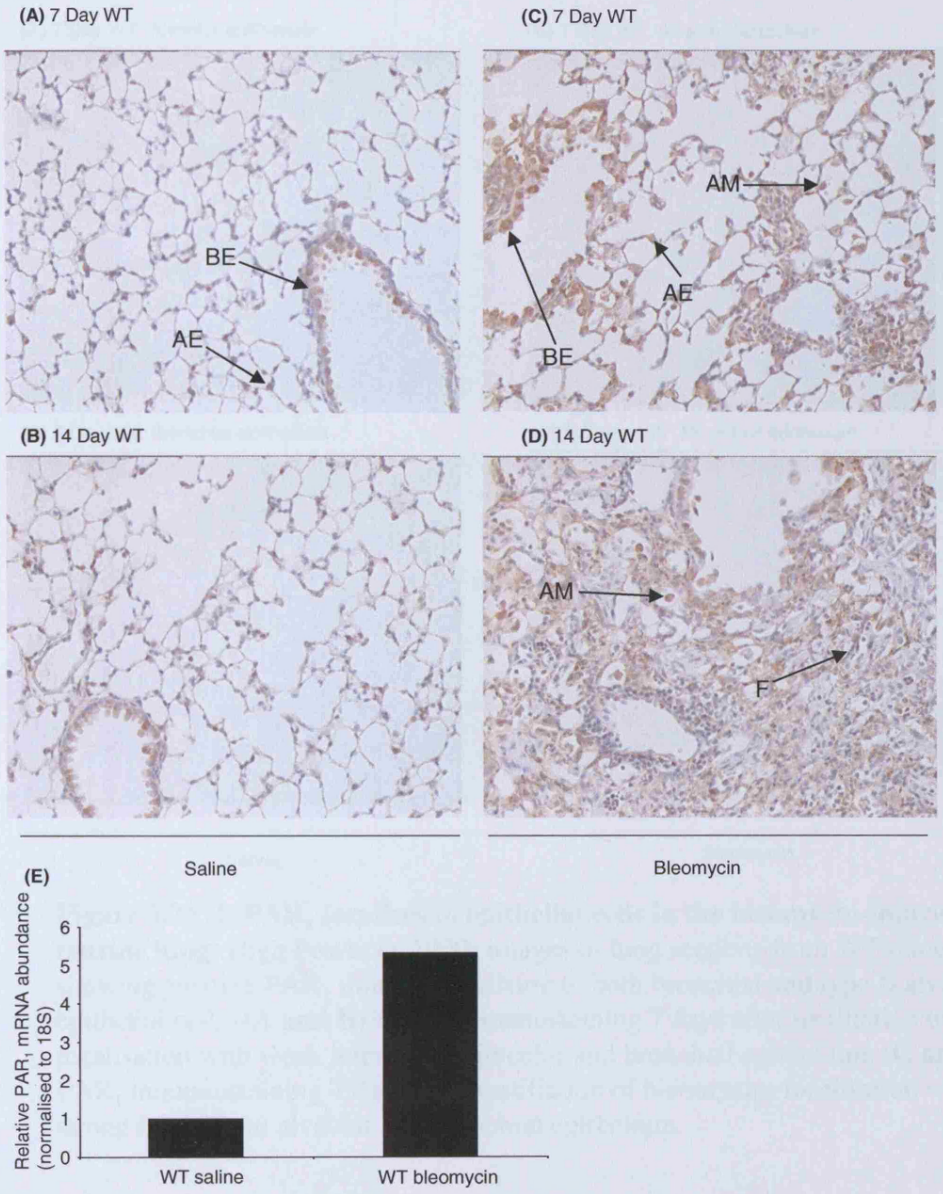


Figure 3.2A.1: PAR_1 expression is increased in response to bleomycin injury: Representative lung tissue sections from WT mice 7 days (**A & C**) and 14 days (**B & D**) after saline and bleomycin instillations, immunostained with anti-SFFL (PAR_1) antibody (original magnification is x 200). (**A & B**) Normal lung architecture after saline instillation: positive immunostaining is confined to the bronchiolar epithelium (BE) with weak positive staining also associated with type II alveolar epithelial cells (AE). (**C**) At seven days after bleomycin injury, the intensity of immunostaining associated with bronchiolar and type II alveolar epithelial cells increased markedly. Strong immunostaining was also apparent on alveolar macrophages (AM) at this time-point. (**D**) At fourteen days after bleomycin injury, immunostaining was again associated with bronchiolar and alveolar epithelial epithelium, but also alveolar macrophages and interstitial spindle-shaped (fibroblast-like) cells (F). (**E**) BALF cell PAR_1 mRNA is increased following bleomycin instillation. Figure shows relative PAR_1 mRNA abundance in pools of lavaged cells 14 days after instillation of saline (10 mice) or bleomycin (3 mice).

Figure 3.2A.2:

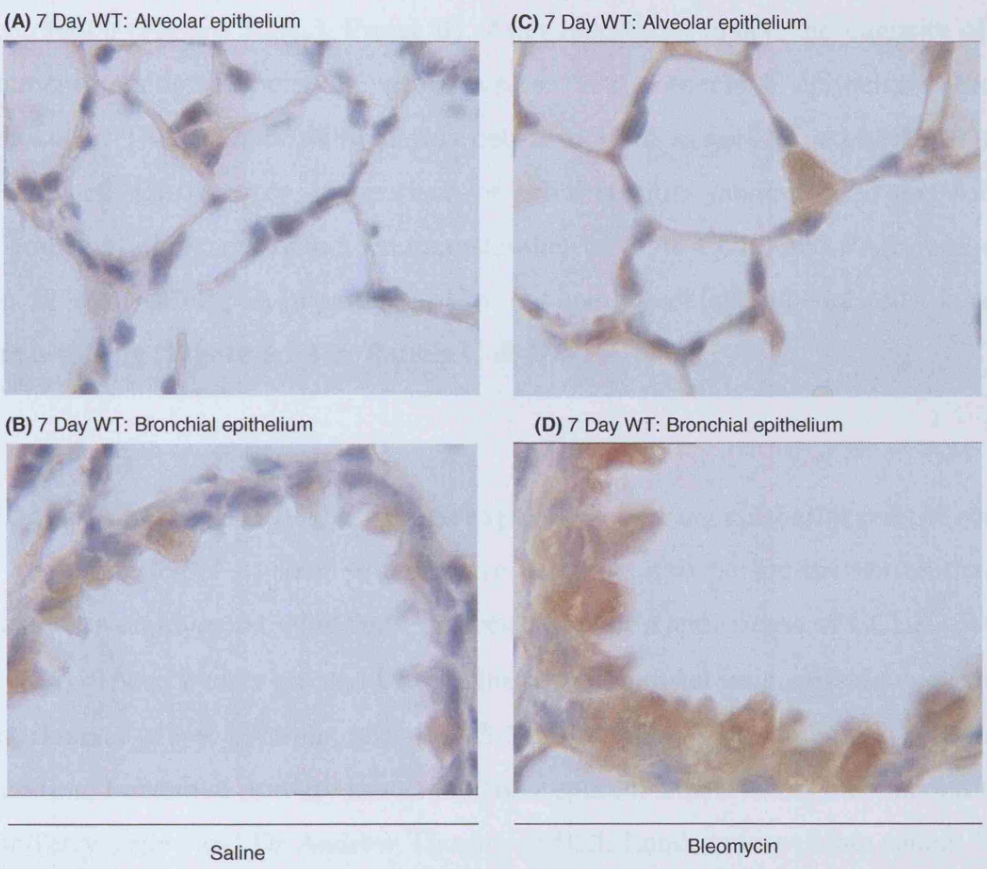


Figure 3.2A.2: PAR_1 localises to epithelial cells in the bleomycin-injured murine lung: High Power (x 1000) images of lung sections from WT mice showing positive PAR_1 immunolocalising to both bronchial and type II alveolar epithelial cells. (**A and B**) PAR_1 immunostaining 7 days after instillation of saline: localisation with weak intensity to alveolar and bronchial epithelium. (**C and D**) PAR_1 immunostaining 7 days after instillation of bleomycin: localisation with strong intensity to alveolar and bronchial epithelium.

3.2A.3. Co-localisation of PAR_1 with CCL2 to lung epithelial cells following bleomycin injury

Epithelial cells have been shown to express CCL2 (Antoniades et al. 1992) in the IPF lung. In this thesis, CCL2 immunolocalisation was examined in sections of mouse lung 6 days following bleomycin. Immunolocalisation of PAR_1 was also performed in parallel in serial sections in order to determine whether CCL2 and PAR_1 co-localised to the same cell types following bleomycin-injury. One might expect that PAR_1 and CCL2 would co-localise to the same cell types if PAR_1 activation induces expression and release of CCL2 from resident lung cells following lung injury.

CCL2 immunoreactivity was faint in sections of mice 6 days following saline instillation (**Figure 3.2A.3, Panel B**). After bleomycin injury, the intensity of CCL2 immunostaining associated with alveolar and bronchial epithelium increased markedly. This immunostaining was cell-associated as well as extracellular matrix-associated. Furthermore, comparison of serial sections demonstrated that following bleomycin injury, prominent immunostaining for both CCL2 and PAR_1 was evident on the same cell types, in particular alveolar and bronchial epithelial cells, in adjacent areas of lung (**Figure 3.2A.3, Panels C & D**).

3.2A.4. Confirmation of PAR_1 gene expression by lung epithelial cells *in vitro*

A series of *in vitro* studies were undertaken to pursue the notion that PAR_1 activation on lung epithelial cells induces expression and release of CCL2, CTGF and TGF-β_1 These studies utilised the murine MLE-15 distal lung epithelial cell line, and the human A549 alveolar and BEAS-2B bronchial lung epithelial cell lines. In addition, I obtained primary human alveolar epithelial cells through collaboration with Dr Terry Tetley and Dr Andrew Thorley (NHLI, London) to validate salient findings obtained in experiments using cell lines.

In the first instance, in order to confirm that the PAR_1 gene was expressed in mouse and human lung epithelial cells, total RNA was extracted from cultured monolayers of unstimulated cells, reverse transcribed, and subjected to PCR analysis using PAR_1-specific primers. Primer design and optimisation and PCR cycling conditions were all optimised in-house (**please see Materials and Methods: 2.3.3 for more details**). Mouse PAR_1 primers amplify a 96bp product corresponding to a specific sequence in the mouse PAR_1 gene; and human PAR_1 primers amplify a 148bp product corresponding to a a specific sequence within the human PAR_1 gene. Mouse lung fibroblasts (MLF) and human foetal lung fibroblast line HFL-1, each known to express PAR_1, were used as positive controls. **Figure 3.2A.4** shows that products of the expected size were obtained for the MLE-15 mouse distal epithelial cell line, the A549 alveolar and BEAS-2B bronchial epithelial cell lines, and for human primary alveolar epithelial cells, confirming expression of PAR_1 by these cells at the mRNA level *in vitro*.

Figure 3.2A.3:

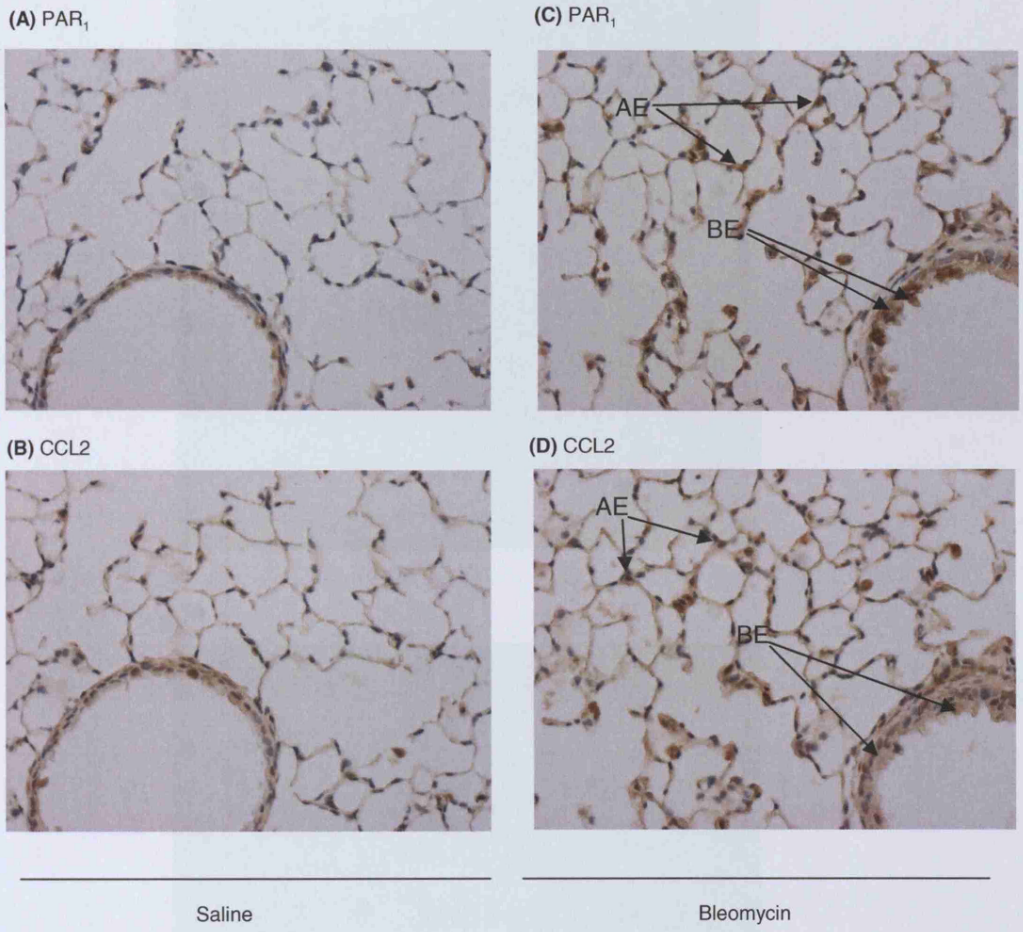
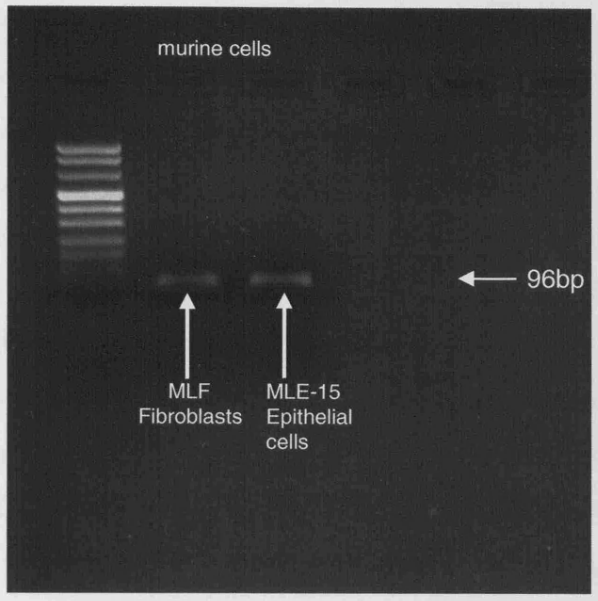


Figure 3.2A.3: CCL2 immunostaining increases following bleomycin, and is co-expressed with PAR_1 on epithelial cells following bleomycin in wild-type mice.

Representative serial lung sections from WT mice 6 days after saline and bleomycin instillations, stained with anti-SFFL (PAR_1) antibody (**A & C**), or anti-CCL2 antibody (**B & D**), depicting immunostaining for both PAR_1 and CCL2 on bronchial (BE) and alveolar epithelial cells (AE) following bleomycin administration. (Original magnification x 200; Sections are 4μm apart).

Figure 3.2A.4: (A)



(B)

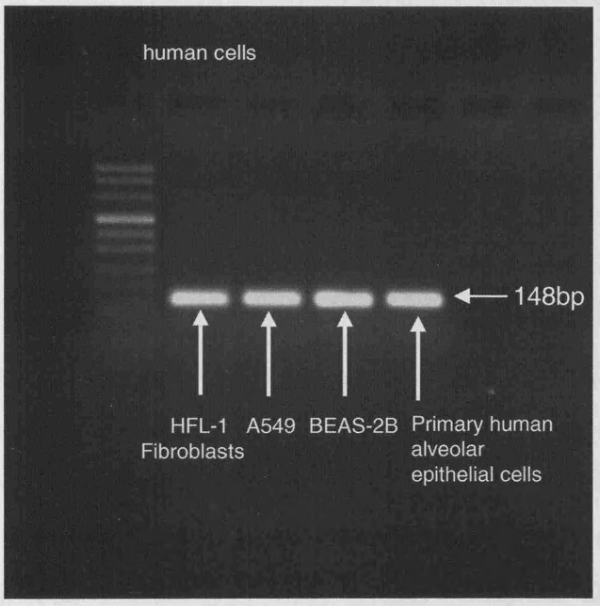


Figure 3.2A.4: Confirmation of PAR_1 gene expression in lung epithelial cells
Images show products amplified by RT-PCR of total RNA isolated from cultured unstimulated monolayers of the indicated cell types. Products were run on a 2% agarose gel. (**A**) 96bp product amplified by murine PAR_1 primers in mouse lung fibroblasts (MLF) and mouse lung epithelial cells (MLE-15). (**B**) 148bp product amplified by human PAR_1 primers in human foetal lung fibroblasts (HFL-1), the human lung alveolar epithelial cell line: A549, the human lung bronchial epithelial cell line: BEAS-2B, and primary human lung alveolar epithelial cells.

3.2A.5. Effect of thrombin and the PAR_1 agonist peptide TFLLR-NH_2 on CCL2 protein release from lung epithelial cells (A549)

In order to establish whether PAR_1 activation on lung epithelial cells influenced CCL2 protein release, CCL2 protein levels were measured in supernatants of cultured monolayers of A549 cells exposed to either thrombin, the PAR_1 agonist peptide TFLLR-NH_2, or the partial-reverse control peptide FTLLR-NH_2, by ELISA. A time-course study demonstrated that a 6h incubation with 10nM thrombin was optimal in producing the maximal fold increase in CCL2 (3.7 ± 0.2; $p<0.01$) of any of the time-points examined relative to unstimulated cells (**Figure 3.2A.5, Panel A**). In a dose response study, thrombin induced a concentration-dependent increase in supernatant CCL2 protein levels with 10nM thrombin producing a 5.8 ± 0.1 fold increase after 6 hours ($p<0.01$) (**Figure 3.2A.5, Panel B**). TFLLR-NH_2 (200μM) induced a similar 6.3 ± 0.2 fold increase, whereas the FTLLR-NH_2 control peptide (200μM) had no effect (**Figure 3.2A.5, Panel C**). (Further data supporting the trend for increased CCL2 protein release following exposure of A549 to thrombin and TFLLR-NH_2 are shown in **Appendix A3**).

3.2A.6. Effect of thrombin and the PAR_1 agonist peptide TFLLR-NH_2 on CCL2 mRNA levels in lung epithelial cells

In order to investigate whether changes observed in CCL2 at the protein level in response to PAR_1 activation were mirrored by changes in CCL2 mRNA levels, mouse (MLE-15) and human epithelial cells (A549 and BEAS-2B) were exposed to TFLLR-NH_2 (200μM) and thrombin (10nM) over a 12 hour time-course. RNA was extracted from cell monolayers with TRIzol reagent, DNAse treated, reverse-transcribed, diluted, and then subjected to real-time PCR with specific murine or human CCL2 and 18S primers (**please see Materials and Methods: 2.3.3 for a detailed description of this methodology and its work-up**).

Exposure to thrombin and TFLLR-NH_2 was associated with maximal increases in CCL2 mRNA levels in A549 cells by 4.1 ± 1.1 and 5.1 ± 0.9 fold; and in BEAS-2B cells by 20.0 ± 2.0 and 34.2 ± 8.4 fold respectively (all $p<0.01$) (**Figure 3.2A.6, Panels A and B**).

Figure 3.2A.5:

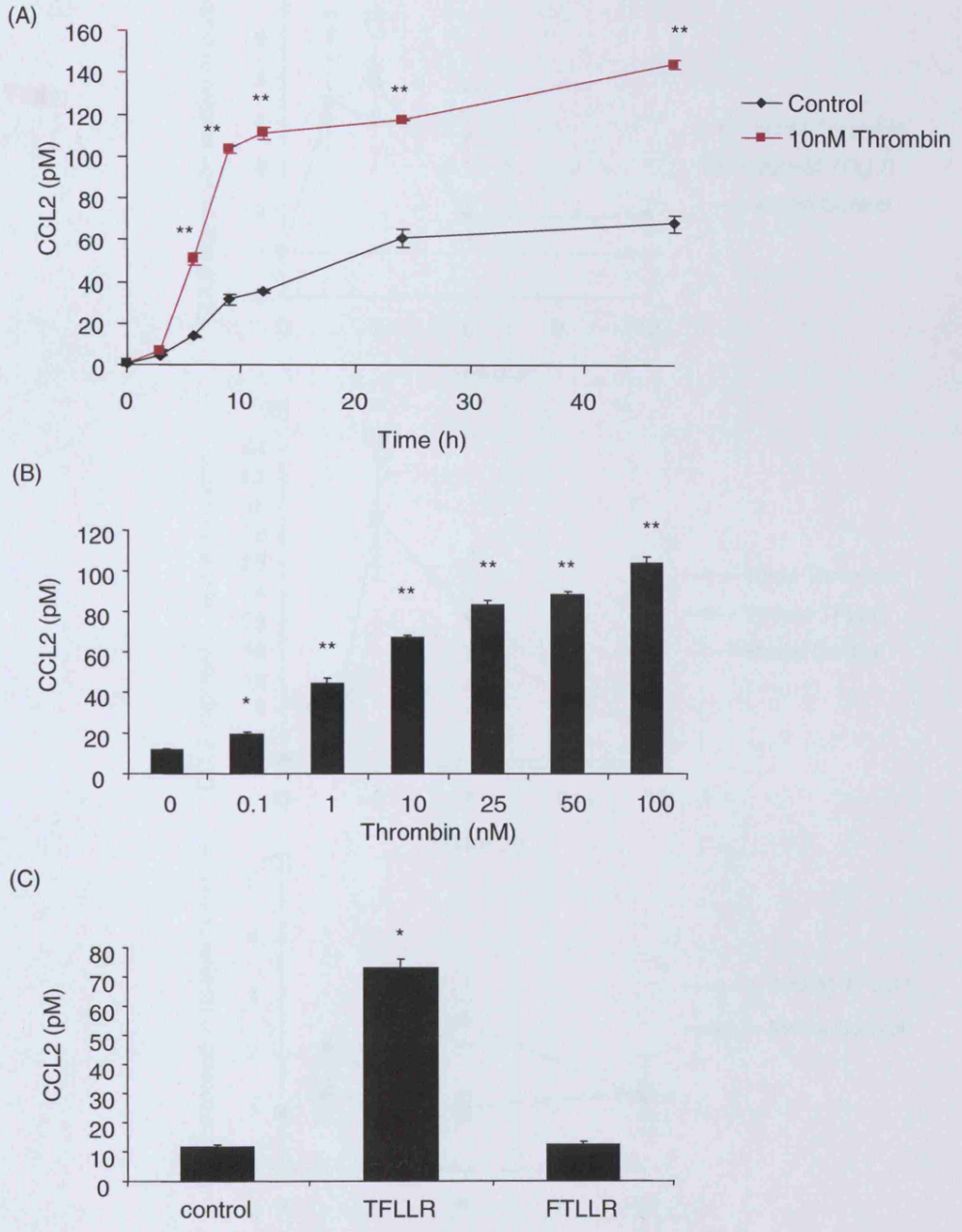


Figure 3.2A.5: Thrombin and the PAR_1 agonist peptide TFLLR-NH_2 promote the release of CCL2 from A549 lung epithelial cells. (**A**) Time course of CCL2 released by A549 in response to 10nM thrombin. Data represent mean ± SEM of 4 replicates. **p<0.01 post-hoc pairwise comparison with control medium at the same time-point (Two Way ANOVA with Student-Newman Keuls post-hoc tests). (**B**) Dose response of thrombin in inducing CCL2 release from A549 at 6 hours. Data represent mean ± SEM of 4 replicates *p<0.01, **p<0.05, comparison of CCL2 levels at each time-point with those exposed to control medium alone (One Way ANOVA with Student-Newman Keuls post-hoc tests.). (**C**) Effect of 200μM TFLLR-NH_2 and FTLLR-NH_2 on CCL2 release. Data represent mean ± SEM of 4 replicates. *p<0.05 comparison with control medium (One Way ANOVA with Dunn's post hoc tests).

Figure 3.2A.6:

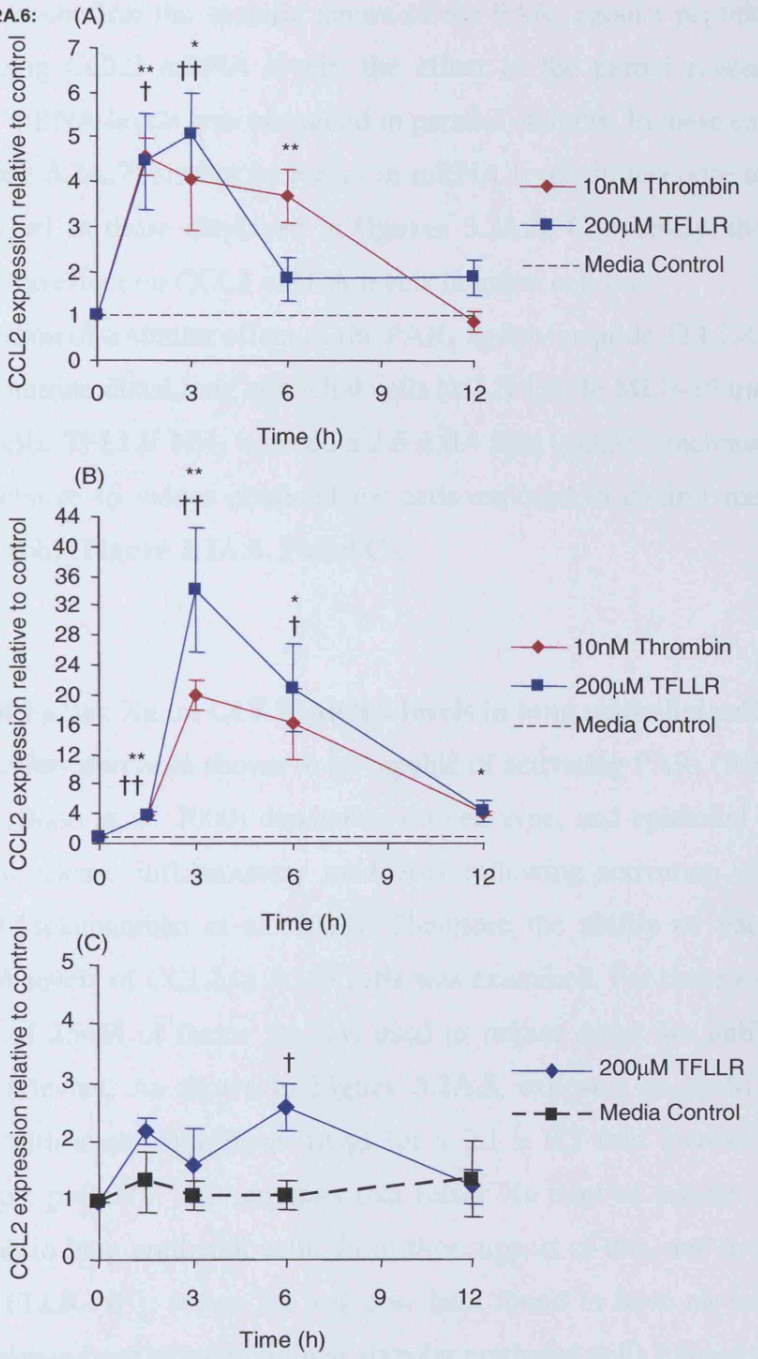


Figure 3.2A.6: The PAR_1 agonist peptide TFLLR-NH_2 and thrombin increase CCL2 mRNA levels in human and murine lung epithelial cells

Figures show mRNA levels (as determined by real Time RT-PCR) of CCL2, normalised to mRNA levels of 18S within each sample, in response to 200μM TFLLR-NH_2 and/or 10nM thrombin and relative to control medium at each time-point. (**A**) A549 cells, (**B**) BEAS-2B cells, (**C**) MLE-15 cells. Data represent mean ± SEM of 3-4 replicates for each timepoint. *$p<0.05$, **$p<0.01$ compared with time-point matched control (thrombin) †$p<0.05$, ††$p<0.01$ compared with time-point matched control (TFLLR-NH_2) (Student's t tests).

In order to confirm the specific nature of the PAR_1 agonist peptide TFLLR-NH_2 in influencing CCL2 mRNA levels, the effect of the partial reverse peptide FTLLR-NH_2 on mRNA levels was examined in parallel cultures. In these experiments (please see **Figure 3.2A.7**) similar increases in mRNA levels in response to TFLLR-NH_2 were observed as those displayed in **figures 3.2A.6**. Conversely, the FTLLR-NH_2 peptide had no effect on CCL2 mRNA levels in either cell line.

Confirmation of a similar effect of the PAR_1 agonist peptide TFLLR-NH2 was also obtained in murine distal lung epithelial cells (MLE-15). In MLE-15 mouse distal lung epithelial cells, TFLLR-NH_2 induced a 2.6 ± 0.4 fold ($p<0.05$) increase in CCL2 mRNA levels relative to values obtained for cells exposed to control media at the same time-point (6h) (**Figure 3.2A.6, Panel C**).

3.2A.7. Effect of Factor Xa on CCL2 mRNA levels in lung epithelial cells

Factor Xa has also been shown to be capable of activating PAR_1 (Riewald et al. 2001) or PAR_2 (Bono et al. 2000) depending on cell type; and epithelial cells have been reported to release inflammatory mediators following activation of PAR_1 or PAR_2 *in vitro* (Asokananthan et al. 2002). Therefore the ability of Factor Xa to influence mRNA levels of CCL2 in A549 cells was examined. For these experiments a concentration of 25nM of factor Xa was used as higher doses are unlikely to be physiologically relevant. As shown in **Figure 3.2A.8**, exposure to 25nM factor Xa was associated with a non-significant trend for a 2.1 ± 0.2 fold increase in CCL2 mRNA levels (ns: $p=0.07$). This suggests that factor Xa may be unable to increase CCL2 expression in lung epithelial cells. In further support of this, and in contrast to thrombin and TFLLR-NH_2, factor Xa was also later found to have no influence on CCL2 protein release from primary human alveolar epithelial cells (please see **Figure 3.2A.13**).

Figure 3.2A.7:

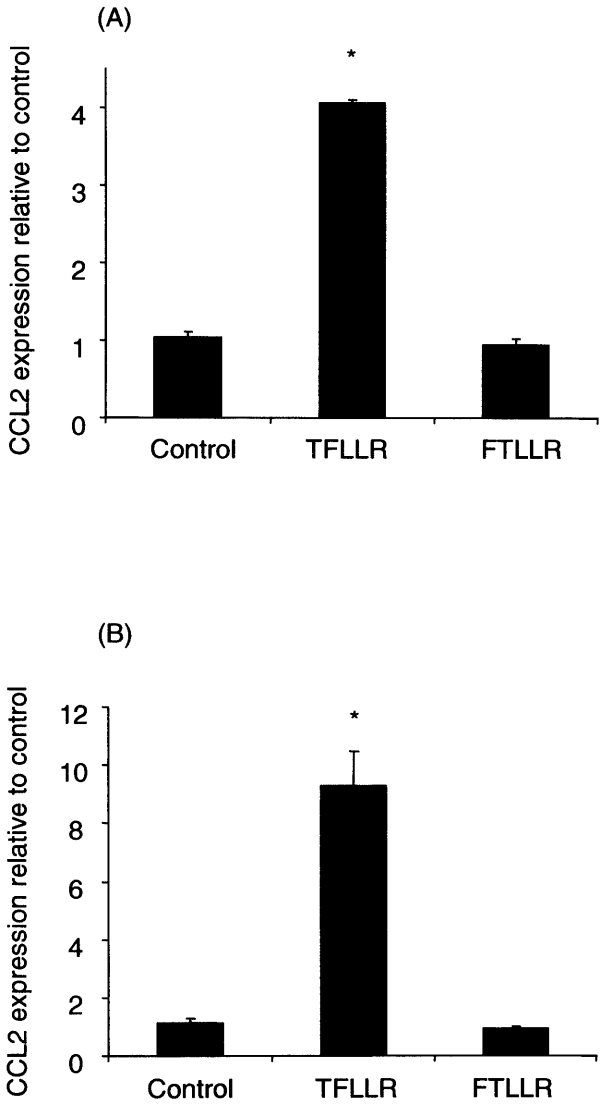


Figure 3.2A.7: Reversal of the first two amino acids of TFLLR-NH_2 completely abrogates its effect on CCL2 mRNA levels in human lung epithelial cells

Figures show relative mRNA levels (as determined by Real Time RT-PCR) of CCL2, normalised to mRNA levels of 18S within each sample, 1.5 h after exposure to control medium, 200μM TFLLR-NH_2 or 200μM FTLLR-NH_2. (**A**) A549, (**B**) BEAS-2B. Data represent the mean ± SEM of 6 replicates. *$p<0.05$ comparison with control (One Way ANOVA with Dunn's post-hoc tests).

Figure 3.2A.8:

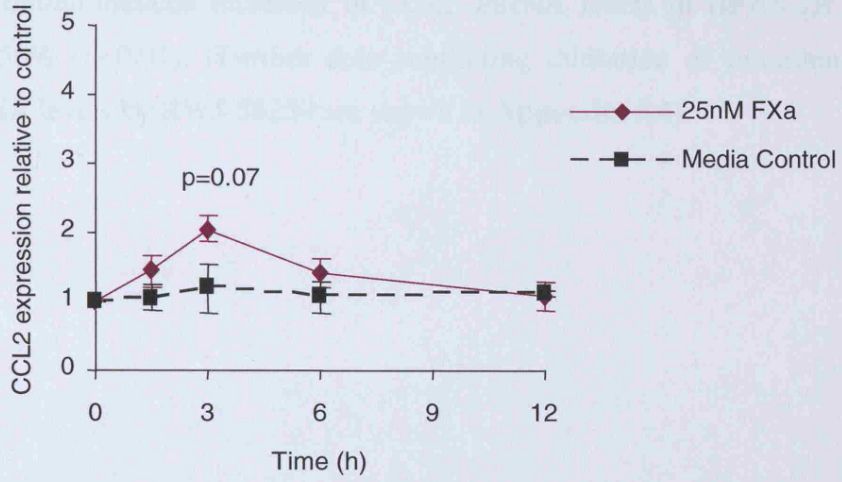


Figure 3.2A.8: Factor Xa has no effect on CCL2 mRNA levels in A549 cells
Figure shows mRNA levels (as determined by Real Time RT-PCR) of CCL2, normalised to mRNA levels of 18S within each sample, in response to 25nM Factor Xa and relative to control medium at each timepoint. $p<0.07$ comparison of FXa-induced mRNA levels with time-point matched control (Student's t test)

3.2A.8. Effect of the specific PAR_1 antagonist RWJ-58259 on thrombin-indued CCL2 mRNA levels in lung epithelial cells

In order to investigate the necessity of PAR_1 for thrombin-induced increases in CCL2 expression, the effect of the specific PAR_1 antagonist RWJ-58259 on CCL2 mRNA levels, in response to thrombin, was examined. This antagonist has been shown to have an IC_{50} of 0.4μM for inhibition of thrombin-induced human platelet aggregation (Damiano et al. 2003). Notably however, in this setting the antagonist was utilised to inhibit a far more rapid PAR_1 response than PAR_1-dependent CCL2

expression. Similar, although slightly higher, concentrations were chosen for experiments described in this thesis in order to maximize the chances of observing an effect. The antagonist was pre-incubated with cells for 20 minutes prior to their stimulation with thrombin. In A549 cells, concentrations of 2 and 6μM RWJ-58259 significantly inhibited thrombin-induced CCL2 mRNA levels, whilst there was a trend for inhibition with 4μM RWJ-58259, although this just failed to reach statistical significance ($p=0.06$). All concentrations of antagonist significantly inhibited the response in BEAS-2B cells (**Figure 3.2A.9**). 6μM RWJ-58259 completely abrogated thrombin-induced increases in CCL2 mRNA levels in A549 cells ($p<0.05$); and blocked thrombin-induced increases in CCL2 mRNA levels in BEAS-2B cells by 63.72 ± 3.50% ($p<0.01$). (Further data supporting inhibition of thrombin-induced CCL2 mRNA levels by RWJ-58259 are shown in **Appendix A4**).

Figure 3.2A.9:

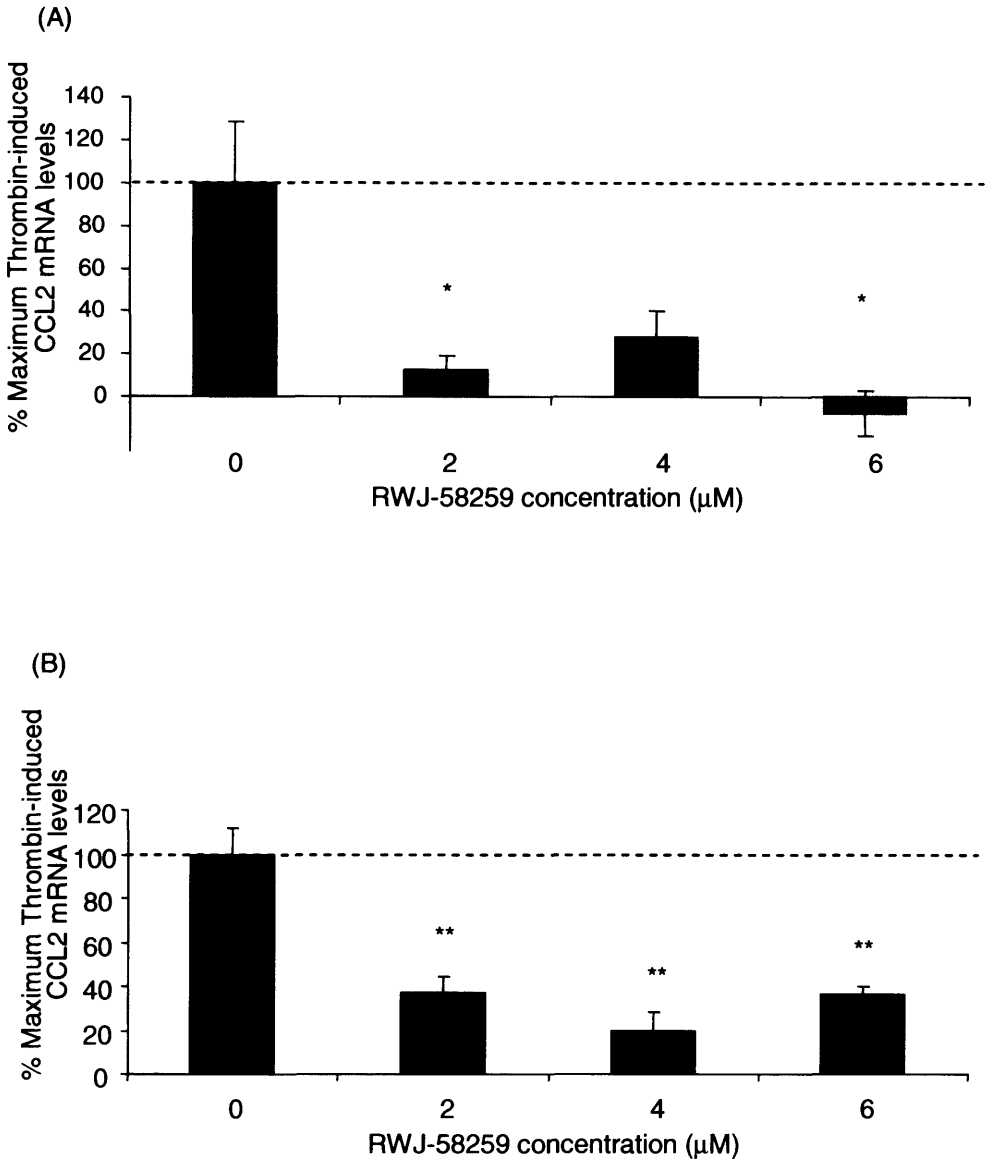


Figure 3.2A.9: The selective PAR_1 antagonist RWJ-58259 inhibits thrombin-induced CCL2 mRNA levels in lung epithelial cells

Figures show effect of RWJ-58259 on the percentage maximum increase in CCL2 mRNA levels in response to 10nM thrombin. (**A**) A549, (**B**) BEAS-2B. Data represent the mean ± SEM of 4 replicates. *$p<0.05$; **$p<0.01$ comparison with maximal thrombin-induced mRNA levels in the absence of RWJ-58259 (One Way ANOVA with Student-Newman Keuls post-hoc tests). Note that RWJ-58259 did not affect baseline CCL2 mRNA levels.

3.2A.9. Effect of the PAR_4 agonist peptide AYPGKF on CCL2 mRNA levels in lung epithelial cells

Thrombin can activate PAR_1, PAR_3, and PAR_4, although PAR_3 does not signal directly (reviewed in (Vergnolle 2000)). Epithelial cells have been reported to release pro-inflammatory mediators following activation of PAR_1, PAR_2, and PAR_4 (Asokananthan et al. 2002), although another report in the literature demonstrates that PAR_4 is undetectable on A549 epithelial cells (Grishina et al. 2005). Thrombin's major cellular receptor is PAR_1; and 10-100 fold higher concentrations of thrombin are required to activate PAR_4 than PAR_1 because of thrombin's far lower affinity for PAR_4 (O'Brien et al. 2001).

Experiments outlined in this thesis show that thrombin increases CCL2 mRNA levels (**Figure 3.2A.6**). It was possible that part of the effect of thrombin might have been mediated by the activation of PAR_4. To determine whether PAR_4 activation influences CCL2 mRNA levels in epithelial cells, A549 cells were exposed to the specific PAR_4 agonist peptide AYPGKF (200μM), which activates PAR_4 in rat (Hollenberg et al. 2004) and human cells (Henriksen & Hanks 2002). As shown in **Figure 3.2A.10, Panel A**, CCL2 mRNA levels, as measured by real-time RT-PCR, were unaltered in A549 cells in response to this peptide.

Real-time RT-PCR was then utilised to examine whether PAR_4 was expressed by A549 cells, and if it was, to assess its abundance relative to PAR_1. PAR_4 specific primers were optimised using cDNA from HFL-1 fibroblasts, and were found to have comparable amplification efficiency as PAR_1 specific primers. Thus it was possible to use these primers simultaneously on paired aliquots of cDNA to evaluate the relative abundance of PAR_1 and PAR_4. In HFL-1, PAR_4 mRNA was detectable but was 1411 times less abundant than PAR_1 ($p<0.01$; experiment based on 3 replicates of unstimulated HFL-1 cells). In A549 cells however, PAR_4 was undetectable in a 45 cycle PCR reaction. Products of the expected size following PCR of HFL-1 and A549 cDNA were obtained (**Figure 3.2A.10, Panel B**) confirming specificity of the primers. These data suggest that A549 cells do not express PAR_4 under the culture conditions employed in this thesis.

Figure 3.2A.10:

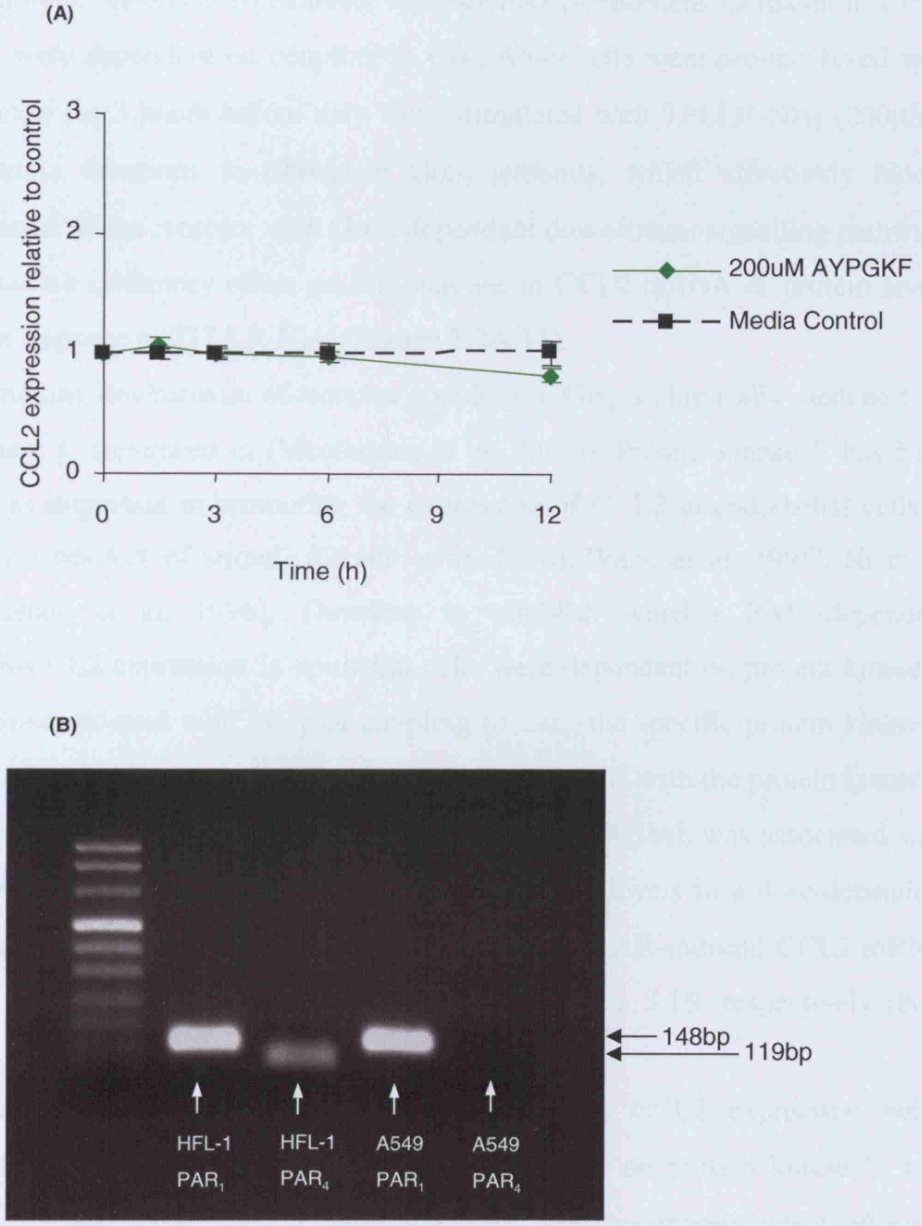


Figure 3.2A.10: The PAR_4 agonist peptide AYPGKF does not influence CCL2 mRNA levels; and PAR_4 is not expressed by A549 cells

(**A**) mRNA levels (as determined by Real Time RT-PCR) of CCL2, normalised to 18S within each sample, in response to 200μM AYPGKF and relative to control medium at each timepoint. Data represent the mean ± SEM of 4 replicates. (**B**) PAR_1 and PAR_4 products amplified by Real-time RT-PCR of total lung RNA from unstimulated HFL-1 or A549 cells. PAR_1 primers amplify a 148bp product. PAR_4 primers amplify a 119bp product.

3.2A.10. Effect of Pertussis Toxin and Protein Kinase C inhibition on PAR_1-dependent increases in CCL2 mRNA in lung epithelial cells

PAR_1 signals by coupling to α_i, α_q, $\alpha_{12/13}$, and $\beta\gamma$ G-protein subunits. Different responses to PAR_1 activation in distinct cell types are achieved by differential coupling to these subunits. To evaluate whether PAR_1-dependent increases in CCL2 expression were dependent on coupling to $G\alpha_i$, A549 cells were pre-incubated with pertussis toxin for 3 hours before they were stimulated with TFLLR-NH_2 (200μM). Pertussis toxin functions to ribosylate $G\alpha_{i/o}$ subunits, which effectively blocks communication of the receptor with $G\alpha_{i/o}$-dependent downstream signalling pathways. Pertussis had no inhibitory effect on the increase in CCL2 mRNA or protein levels observed in response to TFLLR-NH_2 (**Figure 3.2A.11**).

Signalling downstream of receptor coupling to $G\alpha_q$ is classically mediated via protein kinase C (reviewed in (Macfarlane et al. 2001)). Protein kinase C has been implicated as important in promoting the expression of CCL2 in endothelial cells in response to a number of stimuli (Okada et al. 1998),(Wang et al. 1995),(Ni et al. 2003),(Takahara et al. 1996). Therefore to establish whether PAR_1-dependent increases in CCL2 expression in epithelial cells were dependent on protein kinase C and therefore consistent with receptor coupling to $G\alpha_q$, the specific protein kinase C inhibitor Ro-318425 was used. Pre-incubation of A549 cells with the protein kinase C inhibitor Ro-318425 prior to exposure to TFLLR-NH_2 (200μM), was associated with inhibition of TFLLR-induced CCL2 mRNA and protein levels in a dose-dependent manner (**Figure 3.2A.12**). 10μM Ro-318425 blocked TFLLR-induced CCL2 mRNA levels and protein levels by 110.47 ± 2.65% and 76.6 ± 3.1% respectively (both $p<0.01$).

These data are consistent with PAR_1-mediated CCL2 expression being independent of receptor coupling to $G\alpha_i$, but dependent on protein kinase C, and potentially downstream of receptor coupling to $G\alpha_q$. (Further experiments confirming similar effects of pertussis toxin and Ro-314825 are shown in **Appendix A5**).

Figure 3.2A.11:

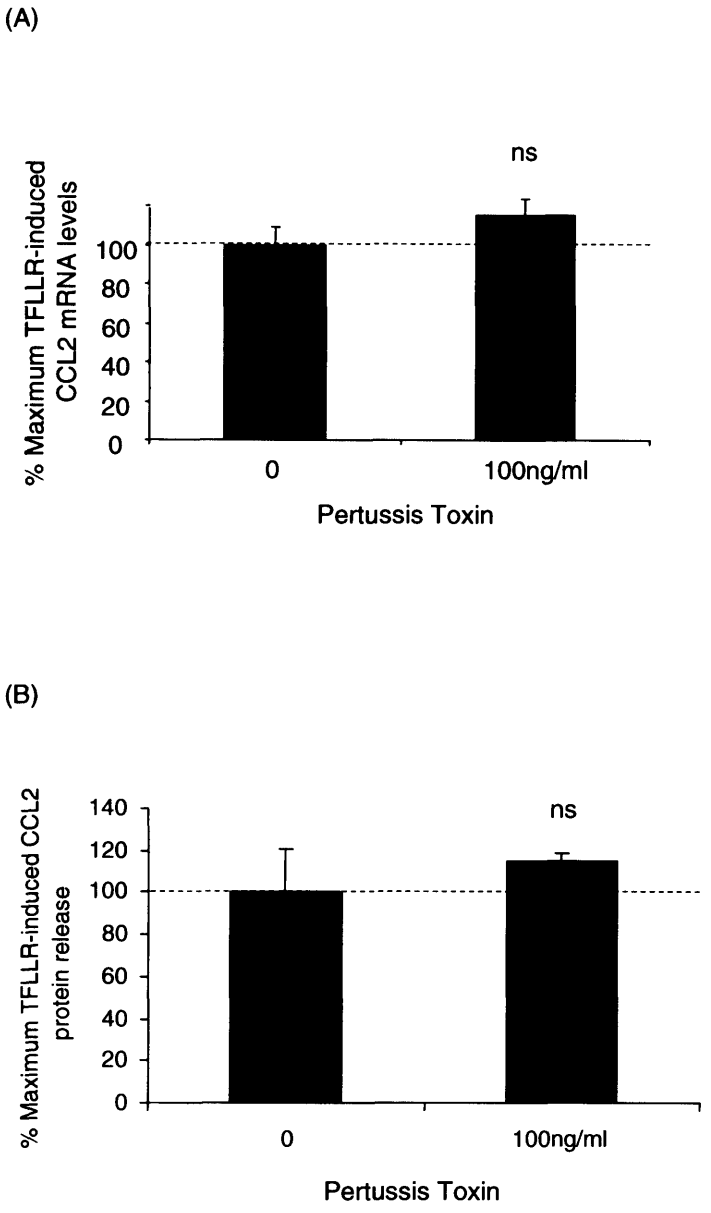


Figure 3.2A.11. Expression of CCL2 in response to TFLLR-NH_2 is Pertussis-insensitive.
(**A**) Effect of pertussis toxin on the percentage maximum increase in CCL2 mRNA levels in response to 200μM TFLLR-NH_2. Data represent the mean ± SEM of 6 replicates. (**B**) Effect of Pertussis toxin on TFLLR-induced CCL2 protein release into culture supernatants, as measured by ELISA. Data represent mean ± SEM of 6 replicates. ns: not significant, comparison of TFLLR-induced CCL2 release in the presence of pertussis to that without pertussis toxin. (Student's t test). Note that pertussis toxin did not affect baseline CCL2 mRNA or protein levels.

Figure 3.2A.12:

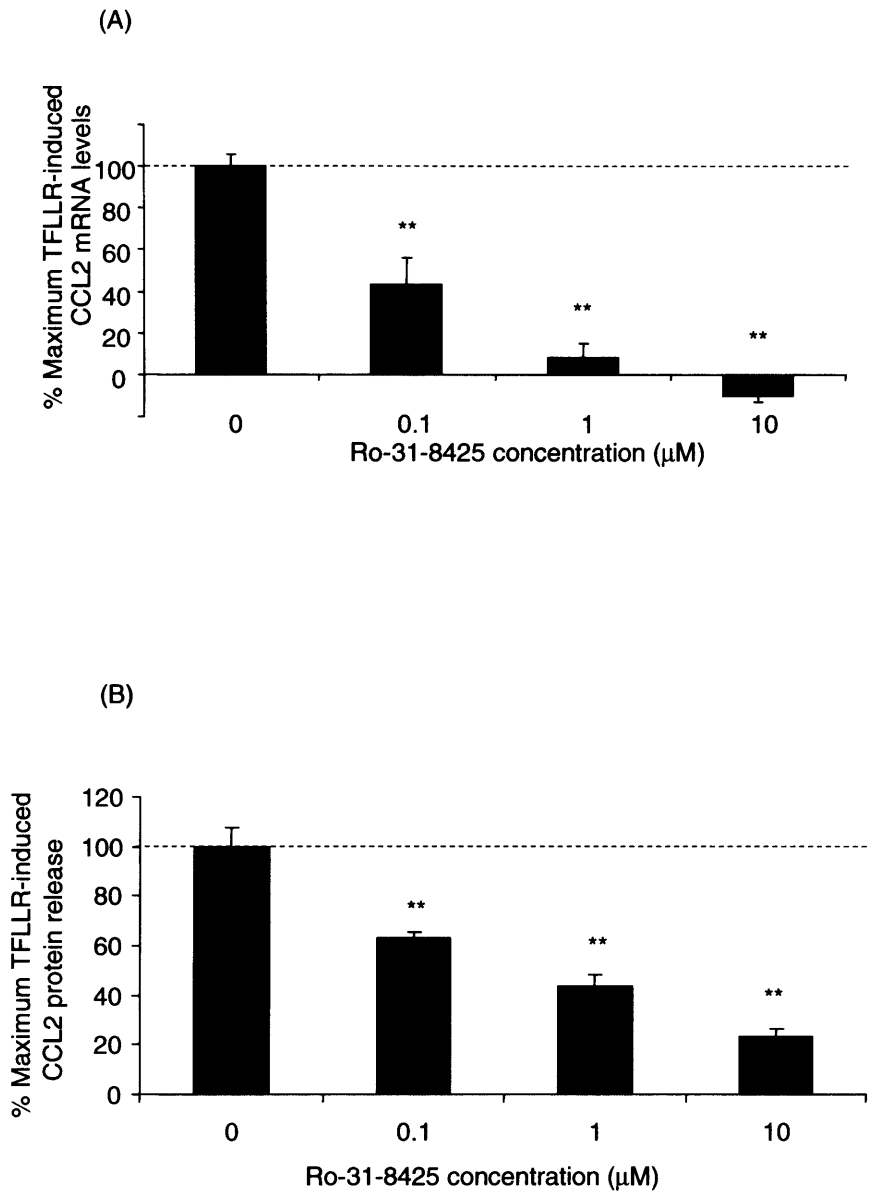


Figure 3.2A.12: TFLLR-induced CCL2 mRNA and protein levels are protein kinase C-dependent.

Figure shows the effect of the PKC inhibitor Ro-318425 on the percentage maximum increase in CCL2 mRNA (**A**), and supernatant protein (**B**) levels in response to 200μM TFLLR-NH_2. Data represent the mean ± SEM of 4 replicates. **$p<0.01$ comparison with maximal TFLLR-induced CCL2 mRNA or protein levels in the absence of Ro-318425 (One Way ANOVA with Student-Newman-Keuls post-hoc tests). Note that Ro-318425 did not affect baseline CCL2 mRNA or protein levels.

3.2A.11. Effect of PAR_1 agonists on CCL2 protein release from *primary* human alveolar epithelial cells.

To establish whether PAR_1 activation influenced CCL2 protein release from *primary* human alveolar epithelial cells, cultured monolayers were incubated for 6h with either thrombin (10nM), Factor Xa (25nM), or TFLLR-NH_2 (200μM), and CCL2 levels measured in the supernatant by ELISA. **Figure 3.2A.13** shows that thrombin increased CCL2 protein levels by 16.0 ± 4.0% ($p<0.05$), whereas TFLLR-NH_2 increased CCL2 protein levels by 18.6 ± 2.9% ($p<0.05$). Factor Xa did not significantly increase CCL2 protein release above the level of that from cells incubated with control media alone. (Further data from two separate experiments, utilising cells obtained from two separate donors, and showing increased CCL2 release from primary alveolar epithelial cells in response to thrombin and TFLLR-NH_2, are presented in **Appendix A6**).

3.2A.12. Quantitation of relative abundance of PAR_1 expression by A549 and BEAS-2B cell lines, and by human primary alveolar epithelial cells.

From the previous results, it is interesting to note that the magnitude of PAR_1-mediated response, in terms of the fold increase in CCL2 mRNA levels following exposure to thrombin or TFLLR-NH_2, was greater in BEAS-2B than in A549 cells. In addition, there was a greater relative increase in CCL2 protein release, following exposure to thrombin or TFLLR-NH_2, in A549 than in primary human epithelial cells. To evaluate whether the magnitude of these responses correlated with the amount of PAR_1 expressed on cells of these types, the relative abundance of PAR_1 mRNA in unstimulated cells in culture was compared using quantitative real-time RT-PCR (**Figure 3.2A.14**). PAR_1 mRNA levels were normalised to 18S mRNA levels within each sample, allowing comparison of relative PAR_1 mRNA abundance across different cell types. Relative to A549 cells, BEAS-2B expressed approximately 50% more PAR_1 mRNA ($p<0.05$); and primary human alveolar epithelial cells expressed approximately 75% less mRNA PAR_1 ($p<0.01$). Thus the magnitude of responses in epithelial cells appear to correlate with the abundance of cellular PAR_1.

Figure 3.2A.13:

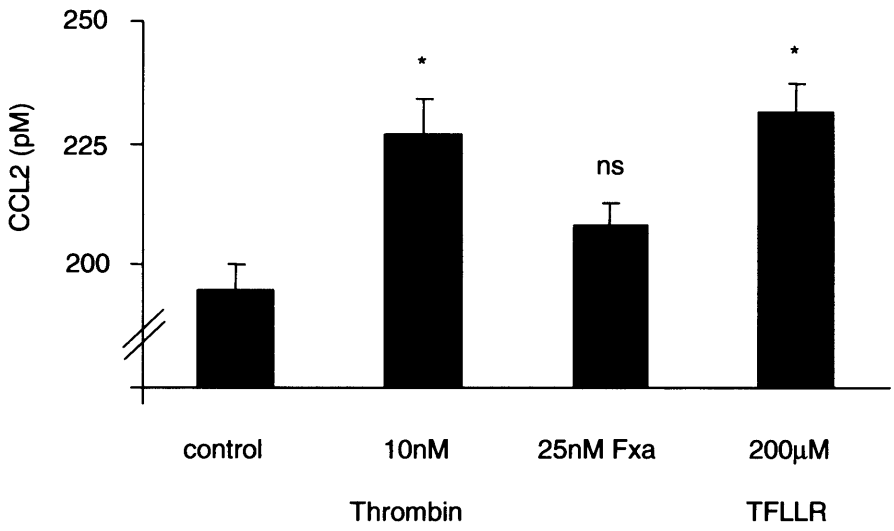


Figure 3.2A.13: Thrombin and the PAR_1 agonist peptide TFLLR-NH_2 promote CCL2 release from human primary lung alveolar epithelial cells. Supernatant CCL2 protein, as measured by ELISA, 6 hours after exposure of primary alveolar epithelial cells to control medium, thrombin (10nM), Factor Xa (25nM), or TFLLR-NH_2 (200μM). Data represent mean ± SEM of 9-10 replicates. ns : not significant, *$p<0.05$ comparison with CCL2 levels for the media control group. (One Way ANOVA with Student-Newman-Keuls post-hoc tests).

Figure 3.2A.14:

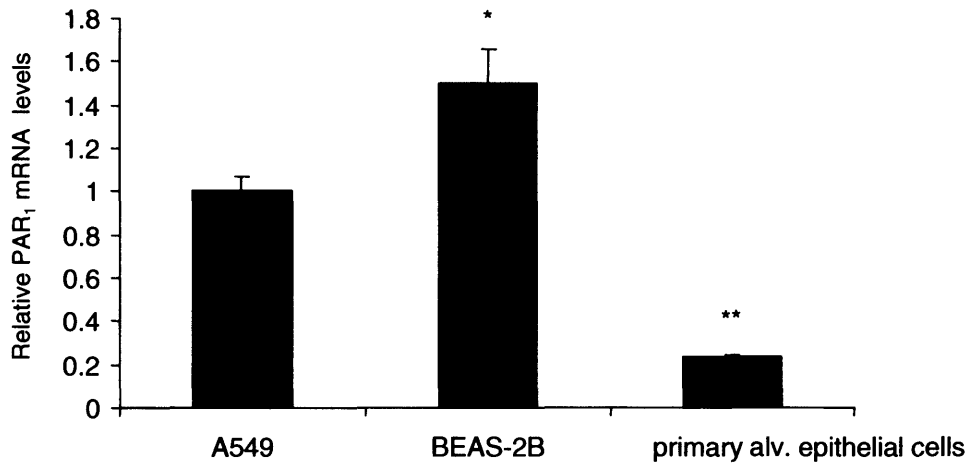


Figure 3.2A.14: Relative expression of PAR_1 (as measured by real-time RT-PCR) by human lung epithelial cells
Data represent normalised mRNA levels in each cell type relative to those in A549 cells, and are the mean ± SEM of 3 replicates. **p<0.01, *p<0.05 comparison with PAR_1 mRNA levels in A549 cells (One Way ANOVA with Student-Newman-Keuls post-hoc tests).

3.2A.13. Influence of conditioned media of epithelial cells exposed to PAR_1 agonists on mononuclear cell chemotaxis

CCL2 is a mononuclear cell chemoattractant. A series of experiments was therefore performed to examine whether conditioned media of lung epithelial cells exposed to PAR_1 agonists infuenced mononuclear cell (THP-1) chemotaxis in transwell migration assays. **Figure 3.2A.15** shows that in the assays employed,

recombinant human CCL2 significantly increased mononuclear cell (THP-1) chemotaxis, and that this effect could be specifically blocked with anti-human CCL2 antibodies.

The ability of conditioned media, generated by exposing monolayers of A549 cells to thrombin, TFLLR-NH_2, and FTLLR-NH_2, to similarly influence THP-1 chemotaxis was then assessed. Unfortunately, these experiments produced inconsistent results when repeated in terms of trends and magnitudes of chemotactic responses. **Figure 3.2A.16** shows two separate experiments showing that conditioned media generated by exposure of epithelial cells to either thrombin (10nM) or factor Xa (25nM) had no significant effect on mononuclear cell chemotaxis. **Figure 3.2A.17** shows three experiments, one of which suggests conditioned media generated by exposure of epithelial cells to TFLLR-NH_2 promoted mononuclear cell chemotaxis above that obtained utlising the control peptide FTLLR-NH_2. However this was not a reproducible finding. Furthermore, there was an apparent trend (not significant) for both TFLLR-NH_2 and FTLLR-NH_2 peptides to promote chemotaxis by themselves to an equivalent extent. This effect was apparrently independent of CCL2, as evidenced by the lack of its inhibition by anti-CCL2 antibodies (**Figure 3.2A.18**).

Therefore, during the course of this thesis it was unfortunately not possible to establish whether CCL2 generated by lung epithelial cells following PAR_1 activation was functional. This would be an important area of further work. However time-pressures precluded further work-up of the methodology for these experiments during the course of this thesis.

Figure 3.2A.15:

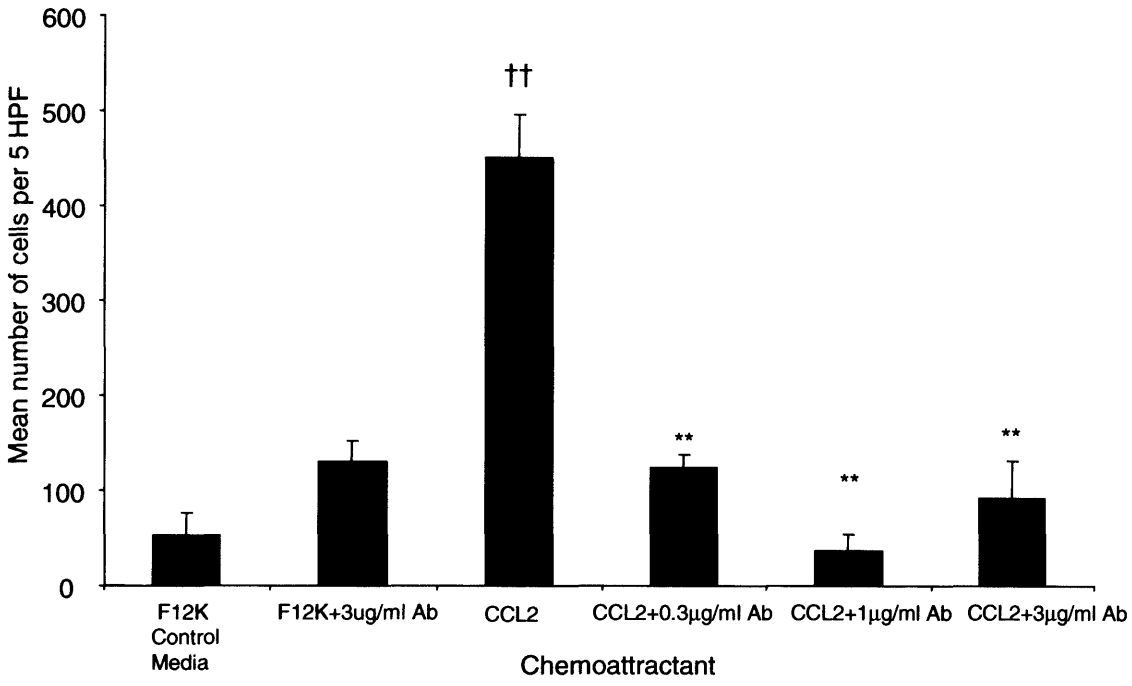
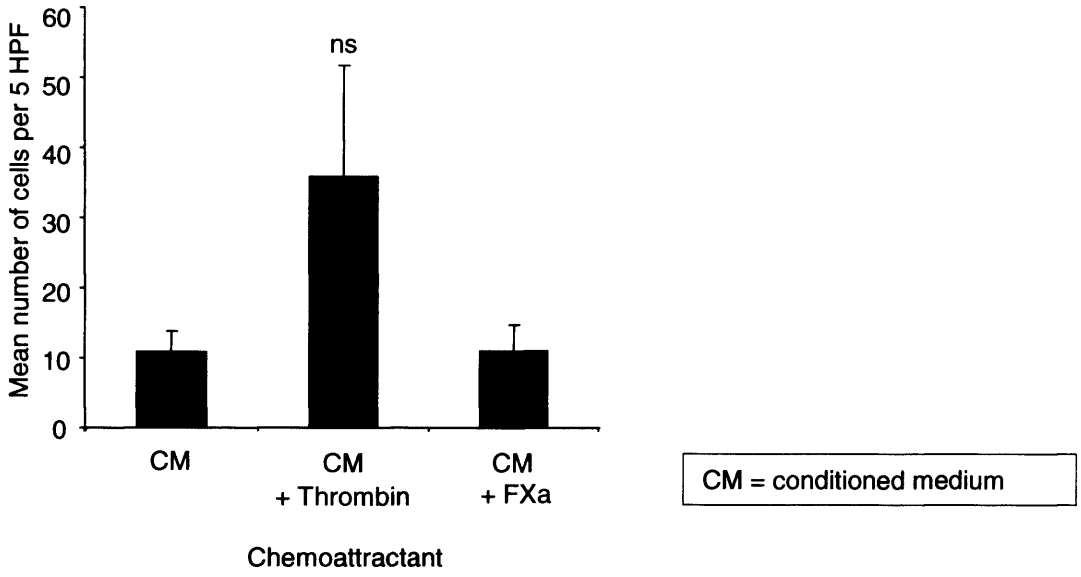


Figure 3.2A.15: CCL2 promotes chemotaxis of THP-1 mononuclear cells. Figure shows the number of THP-1 mononuclear cells (visualised by microscopy per high powered field (HPF)) that have migrated through a porous filter towards the chemoattractants described on the x axis. Recombinant human CCL2 (10ng/ml) induced chemotaxis, and this effect was attenuated by pre-incubation with an anti-CCL2 antibody. Data represent the mean of 5 replicates within each group. ††$p<0.01$ relative to F12K control medium and F12K control medium plus antibody. **$p<0.01$ relative to CCL2 in the absence of antibody (One Way ANOVA with Student-Newman-Keuls post-hoc tests).

Figure 3.2A.16:

(A)



(B)

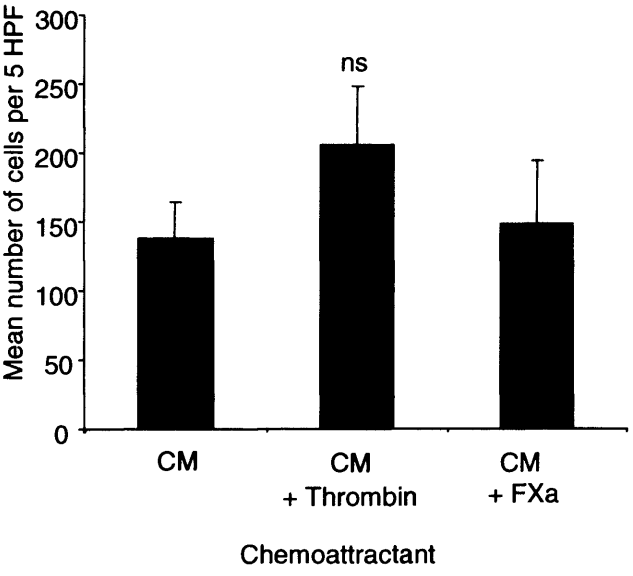


Figure 3.2A.16: Supernatants from epithelial cells exposed to thrombin or factor Xa do not significantly influence THP-1 migration: (A and B) Figures show 2 separate similar experiments comparing the ability of supernatants of A549 cells, exposed to either thrombin (10nM), Factor Xa (25nM) or control medium for 6 hours, to influence migration of THP-1 cells. Data represent the mean of 5 replicates in each group. ns: no significant difference between groups (One Way ANOVA)

Figure 3.2A.17:

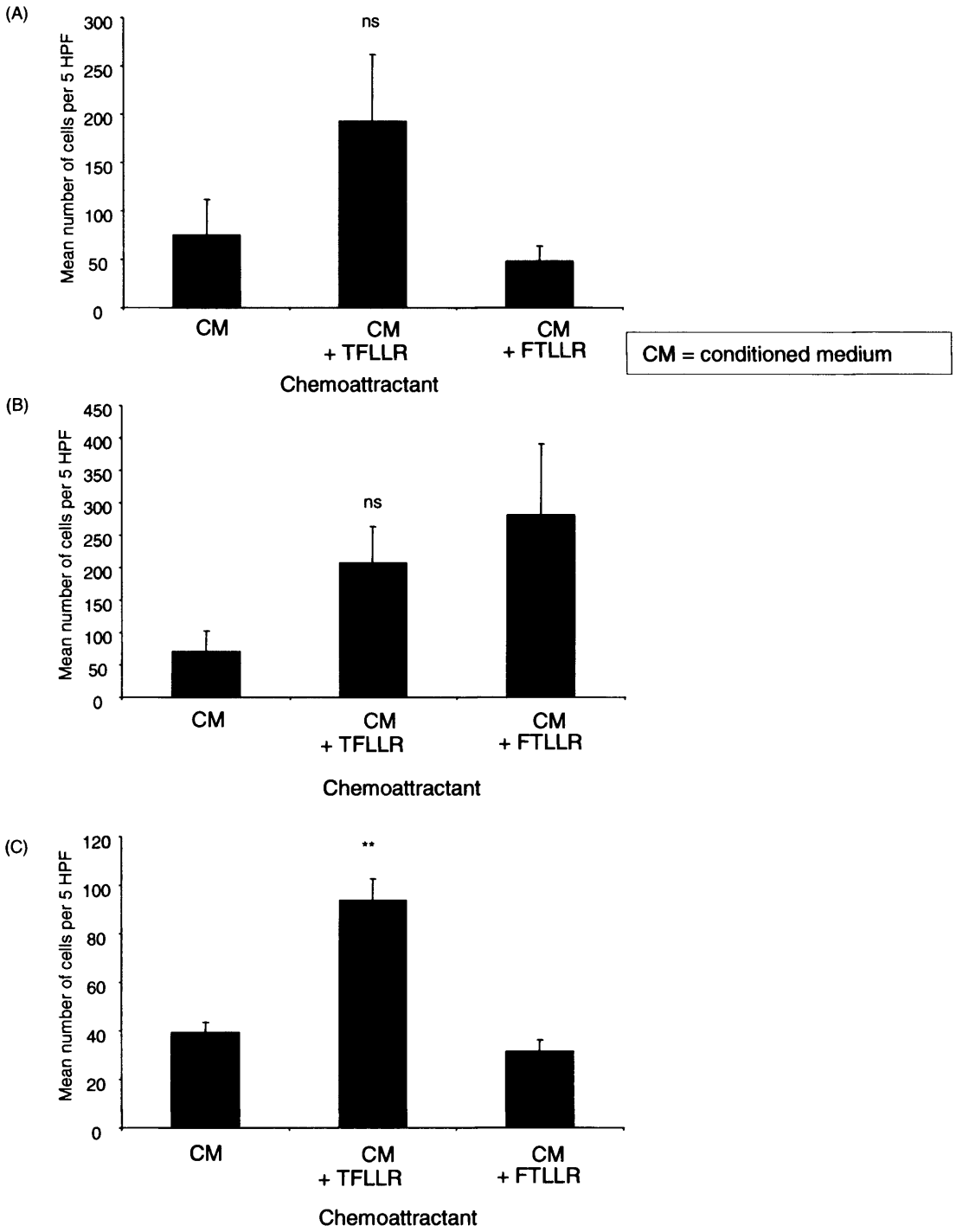


Figure 3.2A.17: Supernatants from epithelial cells exposed to TFLLR-NH_2 (200μM) do not reproducibly influence THP-1 migration: (A-C) Figures show 3 separate similar experiments comparing the ability of supernatants of A549 cells, exposed to control medium, TFLLR-NH_2 (200μM) or FTLLR-NH_2 (200μM) to influence migration of THP-1 cells. Data represent the mean of 5 replicates in each group. ns: no significant difference between groups. ns: no significant difference between groups, **$p<0.01$ relative to control conditioned medium and FTLLR-NH_2 (One Way ANOVA with Student-Newman-Keuls post-hoc tests).

Figure 3.2A.18:

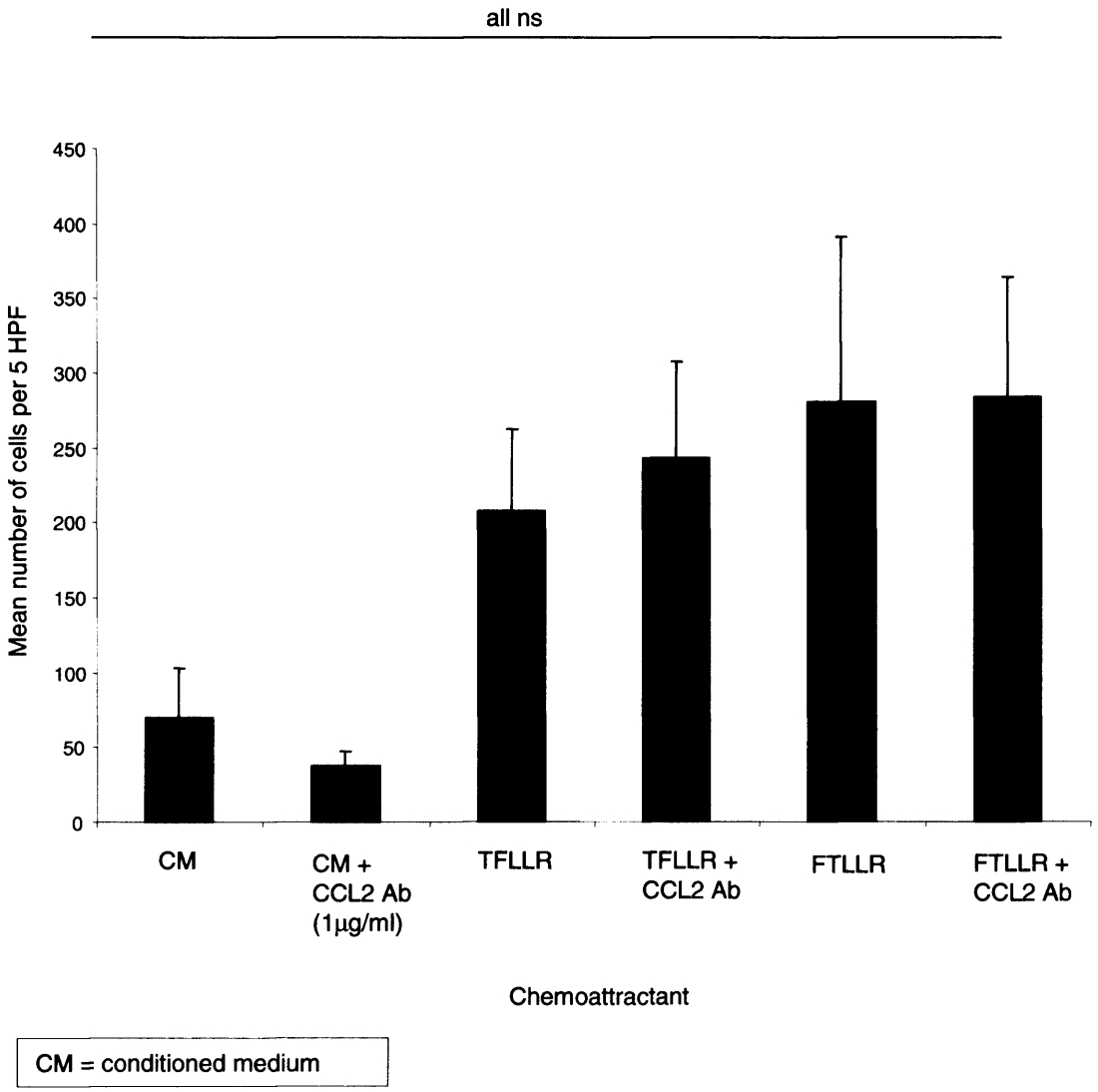


Figure 3.2A.18: Effect of conditioned media, TFLLR-NH_2 and FTLLR-NH_2 peptides on THP-1 migration. Figure shows the effect of conditioned media (CM), TFLLR-NH_2 and FTLLR-NH_2, each with and without anti-CCL2 antibody (1μg/ml), on THP-1 migration. (ns: no statistical differences, One Way ANOVA).

3.2A.14. Summary

- PAR_1 expression increases in the injured murine lung, where it localises to a number of cell types including epithelial cells. PAR_1 is also expressed by lung epithelial cells *in vitro.*
- PAR_1 and CCL2 immunostaining both localise to bronchial and alveolar epithelial cells in adjacent areas of the injured murine lung.
- Exposure of cultured lung epithelial cells to thrombin and TFLLR-NH_2 increases CCL2 mRNA and protein expression.
- CCL2 mRNA levels are not significantly affected by exposure to Factor Xa.
- Thrombin-induced CCL2 mRNA levels are inhibited by the specific PAR_1 antagonist RWJ-58259.
- CCL2 mRNA levels are not significantly affected by exposure to the PAR_4 activating peptide AYPGKF; and A549 epithelial cells do not express PAR_4.
- TFLLR-induced CCL2 mRNA and protein expression is attenuated by protein kinase C inhibition but insensitive to pertussis toxin.
- The relative abundance of PAR_1 mRNA in different epithelial cell types correlates with the magnitude of increased CCL2 protein/mRNA following exposure to PAR_1 agonists.
- Conditioned media generated by exposure of monolayers of lung epithelial cells to PAR_1 agonists does not reproducibly influence mononuclear cell chemotaxis in transwell migration assays.
- Taken together these data suggest that epithelial cells may provide a cellular source of CCL2 expression and release following PAR_1 activation in the injured lung.

3.2B The effect of PAR_1 activation on lung CTGF expression

3.2B.1. Introduction

Elevated tissue levels of CTGF correlate strongly with fibrosis in the bleomycin model of lung injury and in patients with fibrotic diseases of the lung (Allen et al. 1999),(Pan et al. 2001), and other organs (reviewed in (Moussad & Brigstock 2000)). CTGF is constitutively expressed by endothelial cells, myocytes and fibroblasts, but has also been shown to be expressed by epithelial cells (Pan et al. 2001) in the context of lung injury. Following earlier demonstration in this thesis that epithelial cells express PAR_1 in the injured lung, and that epithelial CCL2 expression increased following PAR_1 activation *in vitro*, experiments were performed to examine whether PAR_1 activation also influences CTGF expression by lung epithelial cells.

3.2B.2. Effect of thrombin on CTGF protein expression in lung epithelial cells.

To examine the effect of thrombin on CTGF protein expression in lung epithelial cells, A549 were exposed to either thrombin (25mM) or control media for 4 hours, and the cell layer was lysed in 8M Urea. The concentration and duration of exposure of thrombin were chosen because previous work by Dr Rachel Chambers (Chambers et al. 2000) had previously shown that thrombin increases CTGF protein levels in HFL-1 fibroblasts under similar conditions. The technique of lysing cells in 8M Urea was employed because it was found to produce a far greater protein yield from cell monolayers of epithelial cells than previous attempts using RIPA buffer. CTGF protein was demonstrated in protein extracts of the cell layer by western blotting. Previous attempts at western blotting using RIPA buffer were never associated with any detectable immunoreactive bands in lysates of A549 cells. Western blotting using a polyclonal CTGF antibody showed that the intensity of a 38kDa immunoreactive band was obviously increased in cells exposed to thrombin (**Figure 3.2B.1, Panel A**, and **Appendix A7** for a repeat blot). CTGF protein was also examined by immunocytofluorescence following a similar duration of thrombin exposure using chamber slides (**Figure 3.2B.1, Panel B**). Immunocytofluorescence was similarly increased following exposure to thrombin. These results show that thrombin increases CTGF protein in lung epithelial cells.

Figure 3.2B.1:

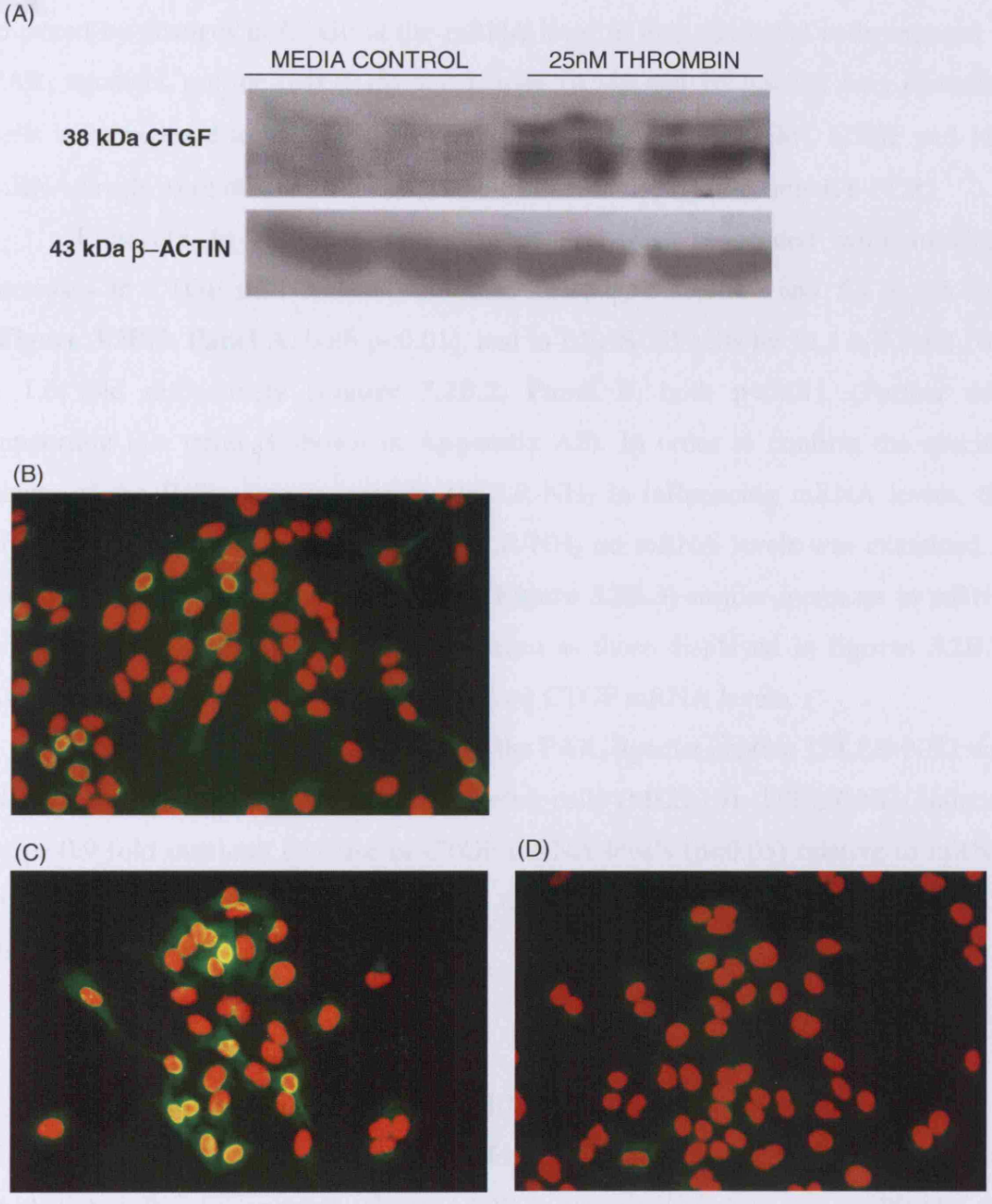


Figure 3.2B.1: Thrombin increases CTGF production in lung epithelial cells (A549). (**A**) Western blot of cell lysates demonstrating that CTGF protein increases after exposure to thrombin (25nM) for 4 hours. Equal protein loading of samples was confirmed by equal β-actin immunoreactivity when the same blot was probed with an anti-β-actin antibody following stripping. (**B-D**) Immunocytofluorescence demonstrating increased CTGF immunoreactivity (yellow) on numerous cells after 4 hours incubation with thrombin (25nM) (**C**) compared to media control (**B**). No immunostaining was seen when a non-immune polyclonal IgG was substituted for the primary anti-CTGF antibody (**D**). nucleii are stained red.

3.2B.3. Effect of the thrombin and the PAR_1 agonist peptide TFLLR-NH_2 on CTGF mRNA levels in lung epithelial cells

In order to determine whether changes in CTGF at the protein level were mirrored by changes in CTGF at the mRNA level in lung epithelial cells exposed to PAR_1 agonists, murine (MLE-15) and human (A549 and BEAS-2B) lung epithelial cells were exposed to TFLLR-NH_2 (200μM) and thrombin (10nM). CTGF and 18S mRNA levels were measured over a 12 hour time-course by real-time RT-PCR.

Exposure to thrombin and TFLLR-NH_2 was associated with maximal increases in CTGF mRNA levels in A549 cells by 5.4 ± 0.7 and 5.1 ± 0.5 fold (**Figure 3.2B.2, Panel A**, both $p<0.01$), and in BEAS-2B cells by 12.1 ± 0.5and 10.0 ± 1.6 fold respectively (**Figure 3.2B.2, Panel B**, both $p<0.01$). (Further data supporting this trend is shown in **Appendix A8**). In order to confirm the specific nature of the PAR_1 agonist peptide TFLLR-NH_2 in influencing mRNA levels, the effect of the partial reverse peptide FTLLR-NH_2 on mRNA levels was examined in parallel. In these experiments (please see **Figure 3.2B.3**) similar increases in mRNA levels in response to TFLLR-NH_2 were seen as those displayed in **figures 3.2B.2**. Conversely, the FTLLR-NH_2 had no effect on CTGF mRNA levels.

Confirmation of a similar effect of the PAR_1 agonist peptide TFLLR-NH2 was also obtained in murine distal lung epithelial cells (MLE-15). TFLLR-NH_2 induced 4.1 ± 0.9 fold maximal increase in CTGF mRNA levels ($p<0.05$) relative to mRNA levels in cells exposed to control media at the same time-point (1.5h) (**Figure 3.2B.2, Panel C**).

3.2B.4. Effect of Factor Xa on CTGF mRNA levels in lung epithelial cells

Factor Xa did not significantly affect CCL2 mRNA or protein levels in lung epithelial cells. In contrast, **figure 3.2B.4** shows that factor Xa (25nM) did significantly increase CTGF mRNA levels, but only by 1.9 ± 0.1 fold ($p<0.01$) in A549 lung epithelial cells. This response was of a much smaller magnitude relative to control media than that observed with either thrombin or TFLLR-NH_2 (**Figure 3.2B.2, Panel A**).

Figure 3.2B.2:

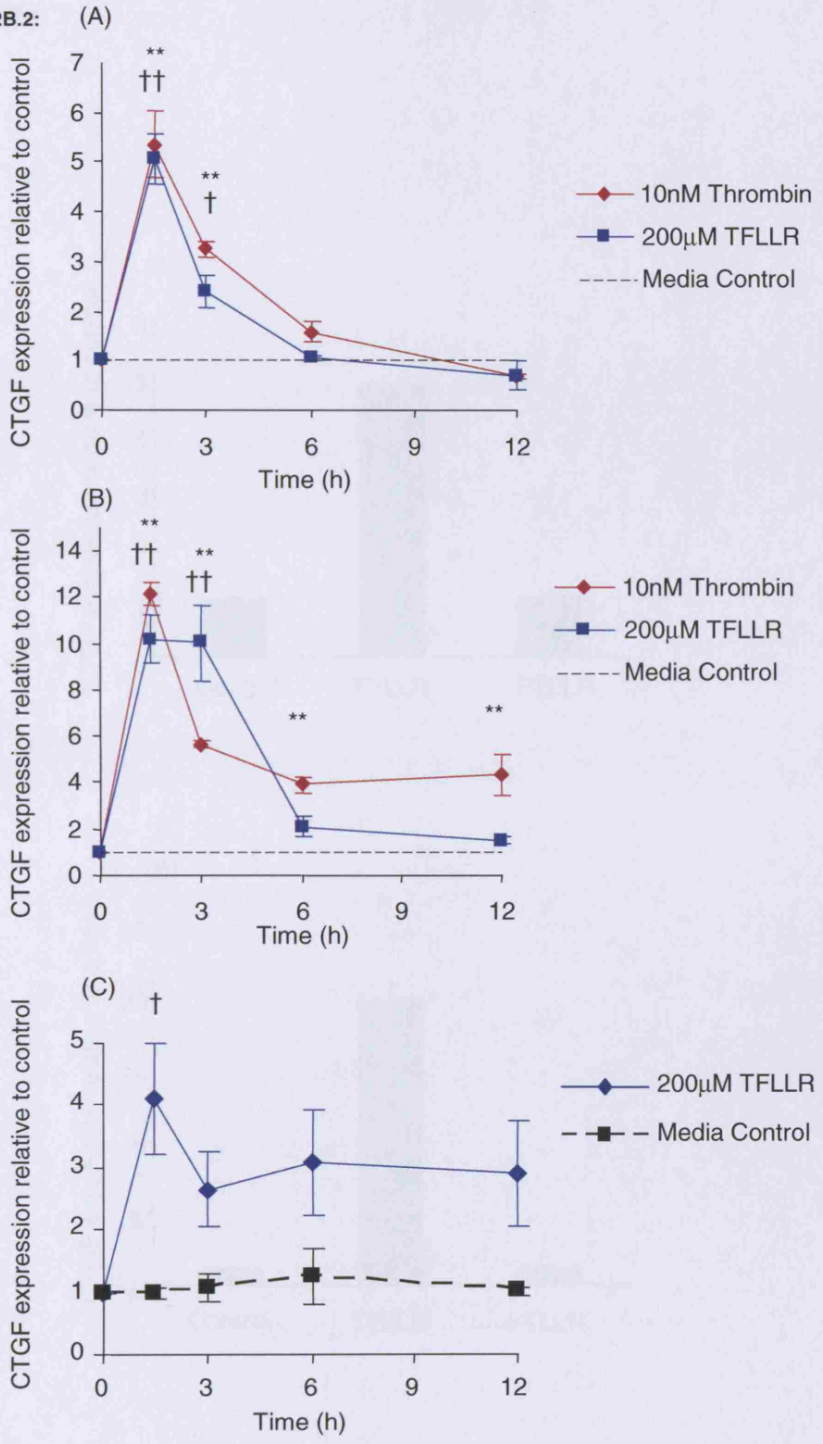


Figure 3.2B.2: The PAR_1 agonist peptide TFLLR-NH_2 and thrombin increase CTGF mRNA levels in human and murine lung epithelial cells

Figures show mRNA levels (as determined by real Time RT-PCR) of CTGF, normalised to mRNA levels of 18S within each sample, in response to 200μM TFLLR-NH_2 and/or 10nM thrombin and relative to control medium at each time-point. (**A**) A549 cells, (**B**) BEAS-2B cells, (**C**) MLE-15 cells. Data represent mean ± SEM of 3-4 replicates for each timepoint. *$p<0.05$, **$p<0.01$ compared with time-point matched control (thrombin) †$p<0.05$, ††$p<0.01$ compared with time-point matched control (TFLLR-NH_2) (Student's t tests).

Figure 3.2B.3:

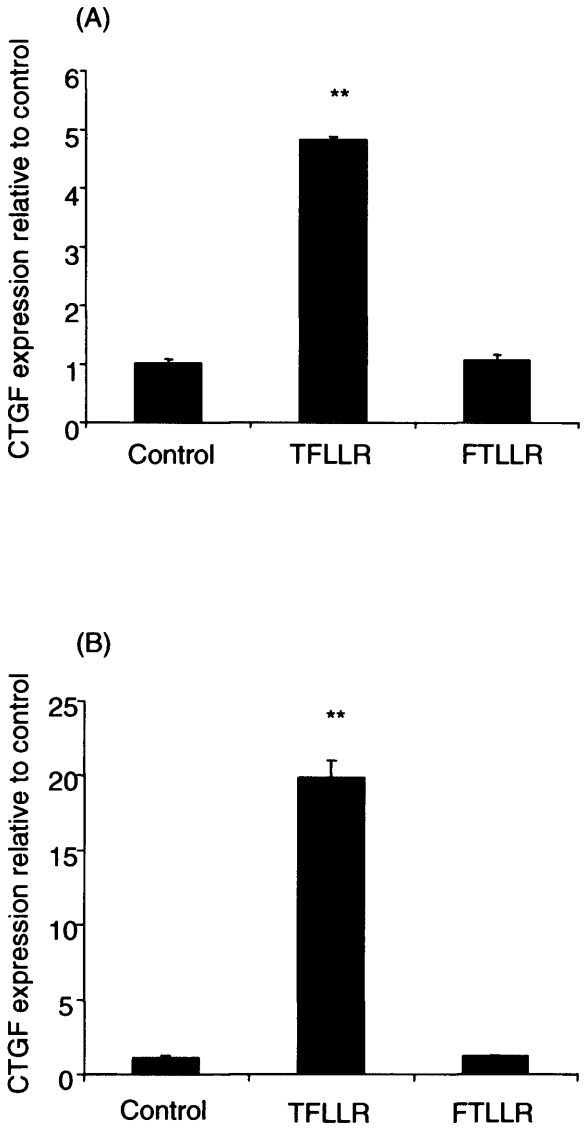


Figure 3.2B.3: Reversal of the first two amino acids of TFLLR-NH_2 completely abrogates its stimulatory effect on CTGF mRNA levels in human lung epithelial cells

Figures show relative mRNA levels (as determined by Real Time RT-PCR) of CTGF, normalised to mRNA levels of 18S within each sample, 1.5h after exposure to control medium, 200μM TFLLR-NH_2 or 200μM FTLLR-NH_2. (**A**) A549, (**B**) BEAS-2B. Data represent the mean ± SEM of 6 replicates. **$p<0.01$ comparison with control. (One Way ANOVA with Dunn's post-hoc tests).

Figure 3.2B.4:

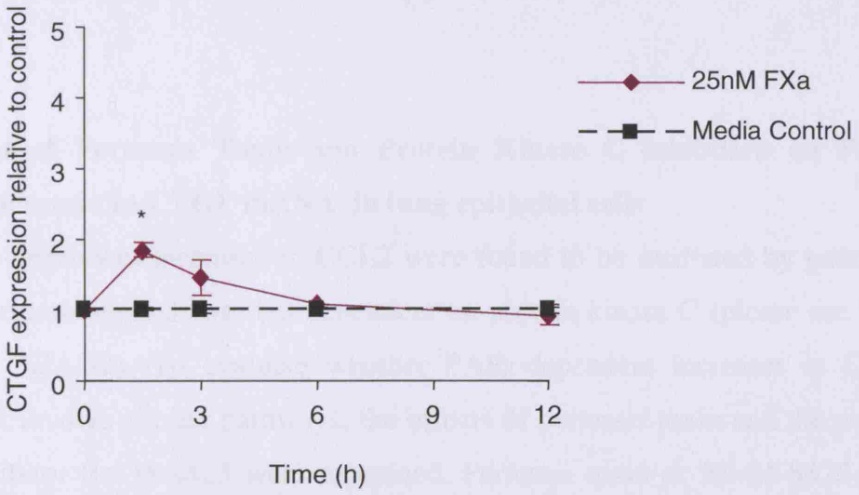


Figure 3.2B.4: Factor Xa exerts a modest effect on CTGF mRNA levels in A549 epithelial cells

Figure shows mRNA levels (as determined by Real Time RT-PCR) of CTGF, normalised to mRNA levels of 18S within each sample, in response to 25nM Factor Xa and relative to control medium at each time-point. *$p<0.05$ comparison of FXa-induced mRNA levels with time-point matched control (Student's t test)

3.2B.5. Effect of the specific PAR_1 antagonist RWJ-58259 on thrombin-indued CTGF mRNA levels in lung epithelial cells

In order to investigate the necessity of PAR_1 for thrombin-induced increases in CTGF mRNA levels, the effect of the specific PAR_1 antagonist RWJ-58259 on CTGF mRNA levels, in response thrombin, was examined. RWJ-58259, at concentrations of 2, 4, and 6μM, blocked increases of CTGF mRNA in response to 10nM thrombin in lung epithelial cells (**Figure 3.2B.5**). At 6μM, RWJ-58259 blocked thrombin-induced increases in CTGF mRNA by by 74.44 ± 2.78 in A549 cells; and by 65.48 ± 2.68 in BEAS-2B cells (both $p<0.01$). (Further data supporting the inhibition of thrombin-induced CTGF mRNA levels are shown in **Appendix A9**).

3.2B.6 Effect of Pertussis Toxin and Protein Kinase C inhibition on PAR_1-dependent increases in CTGF mRNA in lung epithelial cells

PAR_1-dependent increases in CCL2 were found to be mediated by pathways which are pertussis-insenstitive, but dependent on protein kinase C (please see **Figs. 3.2A.11** and **3.2A.12**). To evaluate whether PAR_1-dependent increases in CTGF mRNA might involve similar pathways, the effects of pertussis toxin and the protein kinase C inhibitor Ro-31-8425 were examined. Pertussis toxin or Ro-31-8425 were preincubated with A549 cells *in vitro*, before these cells were stimulated with TFLLR-NH_2 (200μM).

Pertussis had no effect on TFLLR-induced CTGF mRNA levels (**Figure 3.2B.6, Panel A**). The protein kinase C inhibitor Ro-318425 inhibited TFLLR-induced CTGF mRNA levels in a dose-dependent manner (**Figure 3.2B.6, Panel B**). At 10μM, Ro-318425 blocked TFLLR-induced CTGF mRNA levels by 67.19 ± 1.30% ($p<0.01$). (Further data supporting this effect is shown in **Appendix A10**).

These data suggest that PAR_1-dependent CTGF mRNA expression occurs by a pathway that is independent of receptor coupling to $G\alpha_i$, but dependent on protein kinase C, potentially via receptor coupling to $G\alpha_q$.

Figure 3.2B.5:

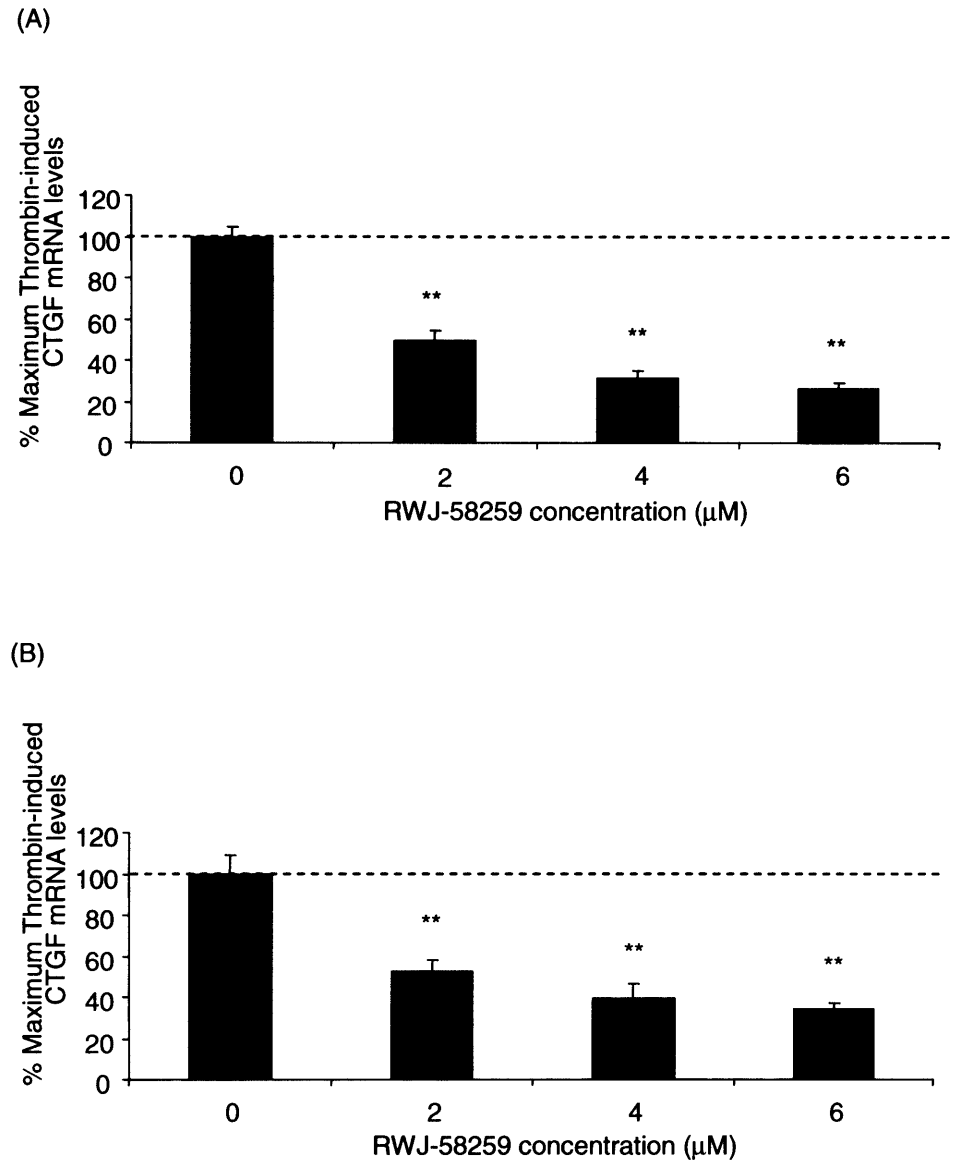


Figure 3.2B.5: The selective PAR_1 antagonist RWJ-58259 inhibits thrombin-induced CTGF mRNA levels in lung epithelial cells

Figures show effect of RWJ-58259 on the percentage maximum increase in CTGF mRNA levels in response to 10nM thrombin. (**A**) A549, (**B**) BEAS-2B. Data represent the mean ± SEM of 4 replicates. **$p<0.01$ comparison with maximal thrombin-induced mRNA levels in the absence of RWJ-58259. (One Way ANOVA with Student-Newman-Keuls post-hoc tests). Note that RWJ-58259 did not affect baseline CTGF mRNA levels.

Figure 3.2B.6:

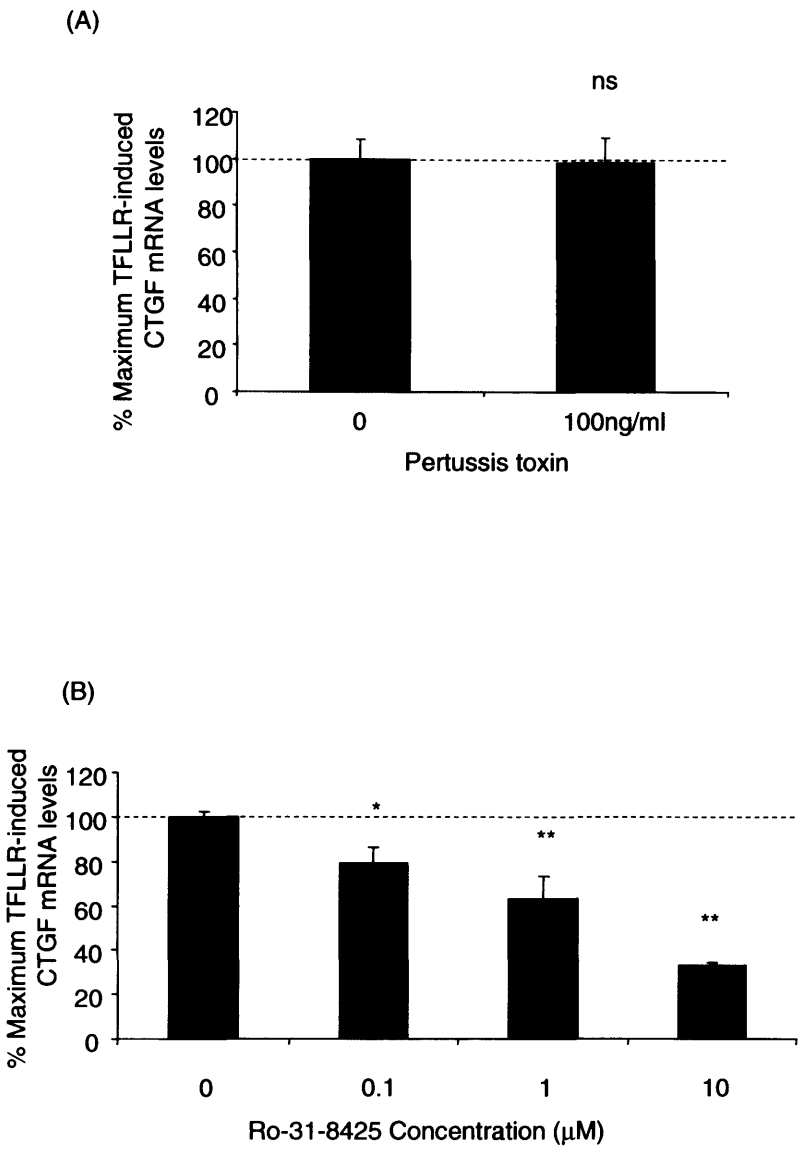


Figure 3.2B.6: Upregulation of CTGF mRNA levels in lung epithelial cells (A549) in response to PAR_1 activation is pertussis-insenstive but protein-kinase C-dependent

(**A**) Figure shows the effect of Pertussis Toxin on the percentage maximum increase in CTGF mRNA levels in response to 200μM TFLLR-NH_2. Data represent the mean ± SEM of 6 replicates. ns : not significant, comparison of TFLLR-induced mRNA levels with pertussis with TFLLR-induced mRNA levels without pertussis (Student's t test).

(**B**) Figure shows effect of the PKC inhibitor Ro-318425 on the percentage maximum increase in CTGF mRNA levels in response to 200μM TFLLR-NH_2. Data represent the mean ± SEM of 4 replicates. *$p<0.05$, **$p<0.01$ comparison with maximal TFLLR-induced mRNA levels in the absence of Ro-318425. (One Way ANOVA with Student-Newman-Keuls post-hoc tests).

Note that Ro-31-8425 and Pertussis toxin did not affect baseline CTGF mRNA levels

3.2B.7. Summary

- Thrombin increases CTGF protein expression by lung epithelial cells *in vitro.*
- Exposure of cultured lung epithelial cells to thrombin and TFLLR-NH_2 increases intracellular CTGF mRNA levels. Responses to thrombin are inhibited by the specific PAR_1 antagonist RWJ-58259. Responses to TFLLR-NH_2 are attenuated by protein kinase C inhibition but are insensitive to pertussis toxin.
- CTGF mRNA levels are significantly but only modestly increased by exposure to Factor Xa.
- Taken together these data suggest that lung epithelial cells may provide a cellular source of CTGF expression and release following PAR_1 activation.

3.3 The effect of PAR_1 activation on lung epithelial cell TGF-β expression

3.3.1. Introduction

Lung epithelial cells are a major source of TGF-β following lung injury in human fibroproliferative lung disease (Kapanci et al. 1995) and the bleomycin model (Azuma et al. 2005a); and TGF-β_1 is widely considered to play a major pro-fibrotic role in the development of fibrosis in these settings (Khalil & Greenberg 1991),(Khalil et al. 1996),(Khalil et al. 1989). Thrombin has previously been shown to increase expression and/or release of TGF-β from vascular smooth muscle cells (Bachhuber et al. 1997), and kidney mesangial and epithelial cells (Yamabe et al. 1997),(Tsunoda et al. 2001),(Shirato et al. 2003). The results outlined in this thesis thus far demonstrate that PAR_1 activation on lung epithelial cells increases the expression of other mediators implicated in lung fibrosis, namely CCL2 and CTGF. The aim of this chapter was to evaluate the effect of thrombin and other PAR_1 agonists on epithelial cell release and expression of TGF-β.

3.3.2. Optimisation of the Mink Lung Epithelial Cell Bioassay for measuring total TGF-β levels

TGF-β in cell culture supernatants was measured using a mink lung epithelial cell bioassay. This assay was originally set up by Abe *et al* (Abe et al. 1994), and utilizes the ability of TGF-β to specifically induce PAI-1 expression. Mink lung epithelial cells were originally stably transfected with an expression construct containing a truncated PAI-1 promoter fused to the firefly luciferase reporter gene. Exposure of these cells to TGF-β results in a dose-dependent increase in luciferase activity in cell lysates. Importantly, this promoter fragment was shown to be only minimally influenced by other known inducers of PAI-1 expression such as bFGF, PDGF-BB, rIL-1α, and EGF. Hence the assay is highly specific for TGF-β.

Following cellular synthesis, TGF-β is released as the biologically inactive latent-TGF-β, which comprises a complex of a latency-associated peptide (LAP) and a 25kDa mature TGF-β dimer. TGF-β activity can potentially be controlled at a

number of levels, such as protein expression and release, sequestration in the extracellular matrix and activation. Since this thesis focused on the ability of PAR_1 activation to promote expression and release of pro-fibrotic mediators, the effect of PAR_1 on TGF-β *activation* was not addressed. The mink lung epithelial cell bioassay actually measures TGF-β activity. Therefore, in order to measure levels of total TGF-β, culture supernatants were spun free of cells and then heated for 10 min at 80°C. This process causes dissociation of the LAP from mature TGF-β dimer, thereby activating the pool of latent TGF-β.

To ensure that PAR_1 activators present in cultured supernatants did not interfere with the MLEC assay, TGF-β_1 standard curves were additionally performed in the presence of 25nM thrombin and 200μM TFLLR-NH_2 and compared with the TGF-β_1 standard curve obtained without these additives. Thrombin and TFLLR-NH_2 had no effect on the measurement of TGF-β_1 standards, and hence this assay is not affected by their presence in culture supernatants **(Figure 3.3.1, Panel A)**. To confirm that the measurable luminescence induced by supernatants of epithelial cells exposed to thrombin was ascribable to TGF-β activity, the pan-specific TGF-β antibody 1D11 *(RnD systems)* was employed. Pre-incubation of samples for 20min at 37°C with 100μg/ml 1D11 completely blocked thrombin-induced luminescence **(Figure 3.3.1, Panel B)**.

3.3.3. Effect of Thrombin and Factor Xa on total TGF-β release from lung epithelial cells

In A549 epithelial cells, thrombin and factor Xa increased supernatant total TGF-β in a concentration- and time-dependent manner **(Figures 3.3.2 and 3.3.3)**. (Further experiments validating these responses are shown in **Appendix A11** and **Appendix A12**. In **Appendix A13**, two separate experiments are described showing that factor Xa and TFLLR-NH_2, but not thrombin, increased supernatant total TGF-β in primary human alveolar epithelial cells.)

Figure 3.3.1:

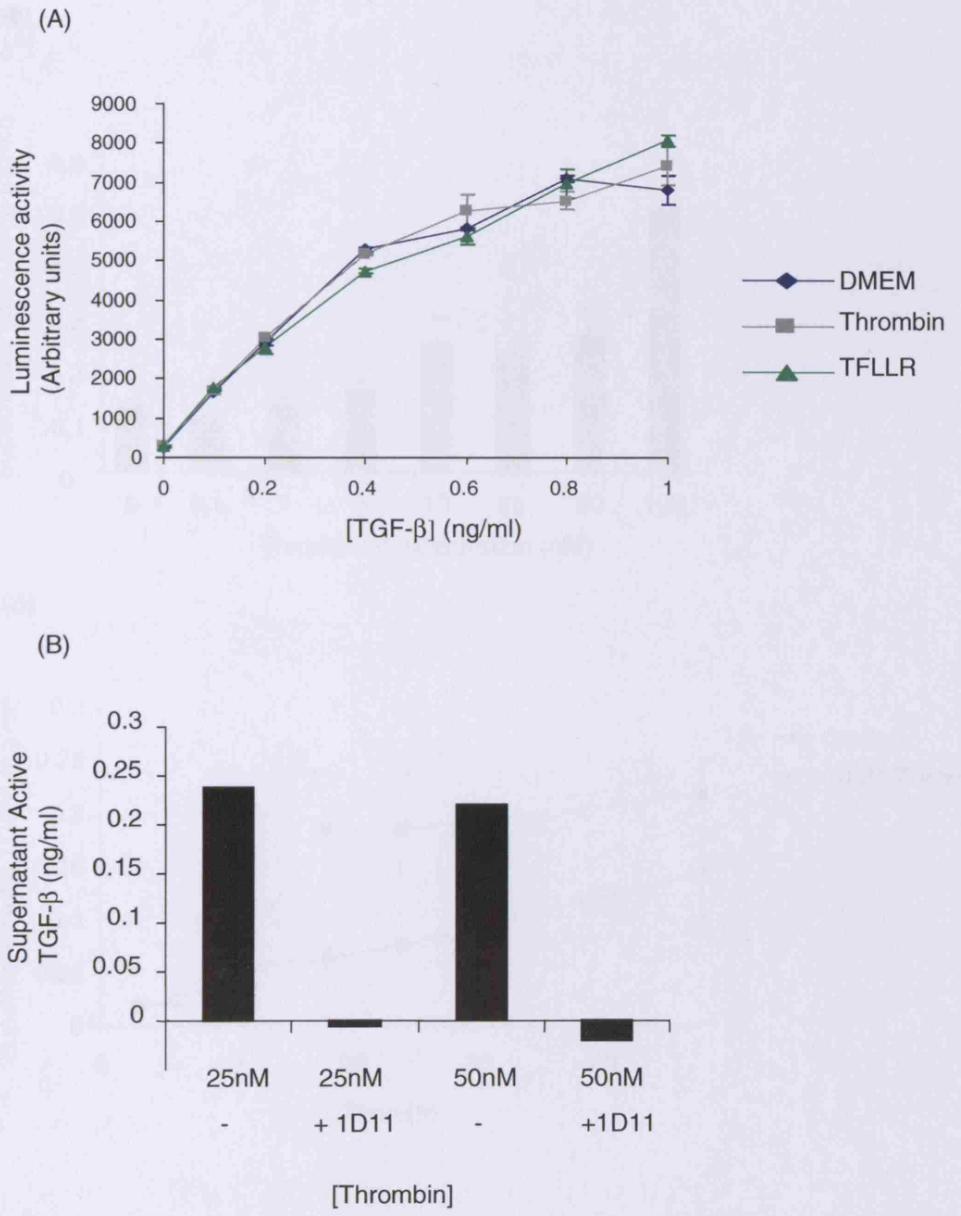


Figure 3.3.1: Validation of the mink lung epithelial cell bioassay for measuring TGF-β activity in supernatants of human epithelial cells exposed to PAR_1 activators. (A) Standard curves of porcine TGF-β_1 in DMEM media alone, and in DMEM with added thrombin (25nM) and TFLLR-NH_2 (200μM): Measurable TGF-β activity (luminescence) is unaffected by the presence of thrombin or TFLLR-NH_2. (**B**) Measurable TGF-β activity in response to thrombin (25nM and 50nM) is abrogated by the pan-specific TGF-β antibody 1D11 (100μg/ml).

Figure 3.3.2:

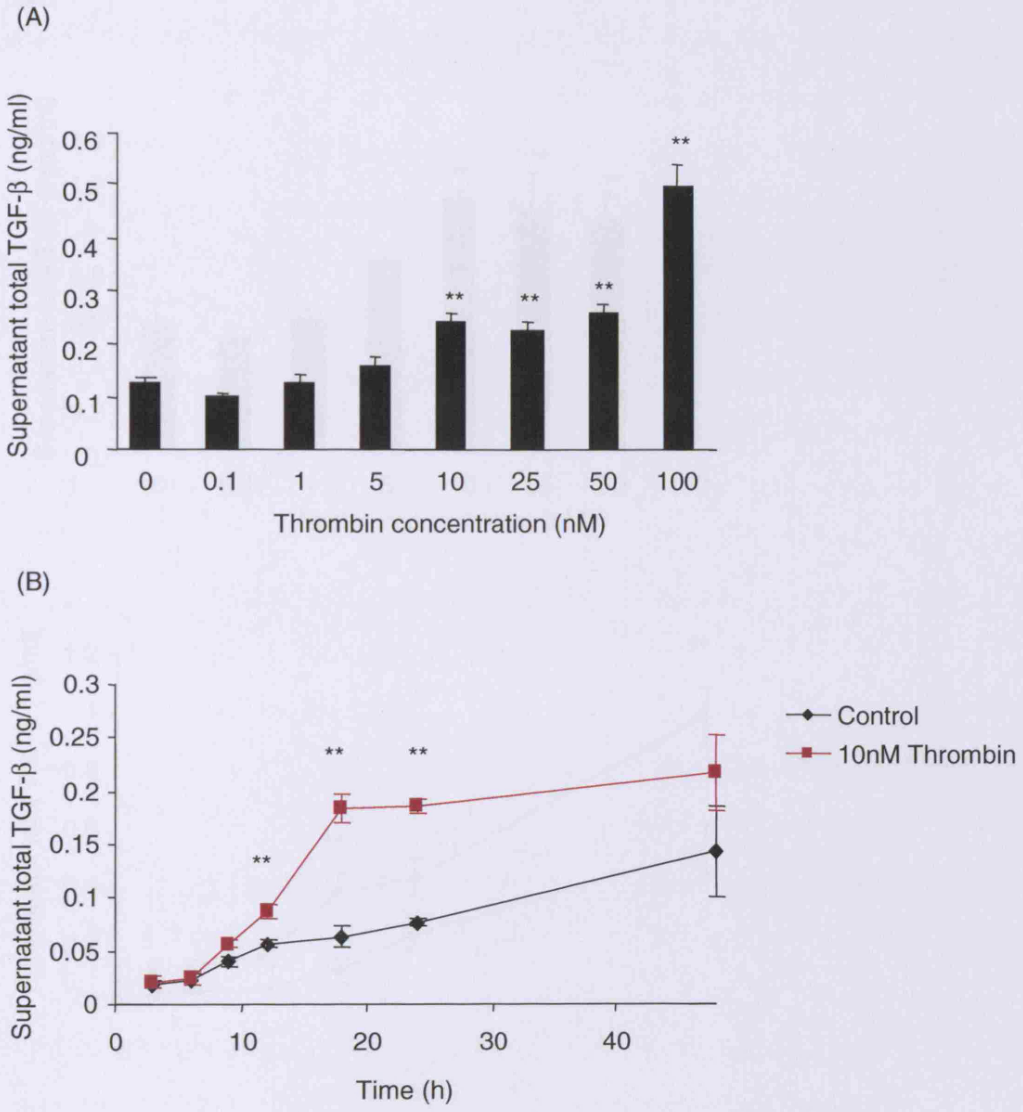


Figure 3.3.2: Thrombin increases total TGF-β release into lung epithelial cell (A549) culture supernatants. (**A**) Thrombin dose response on supernatant total TGF-β levels. Data represent the mean ± SEM of 3-4 replicates. **p<0.01 relative to control (One Way ANOVA with Student-Newman-Keuls post-hoc tests). (**B**) Time course of total TGF-β levels in response to media control or 10nM thrombin. Data represent the mean ± SEM of 3-4 replicates. **p<0.01 comparison with control medium at the same time-point (Two Way ANOVA with Student-Newman-Keuls post-hoc tests). All data were extrapolated from a parallel TGF-β standard curve included for each experiment.

Figure 3.3.3:

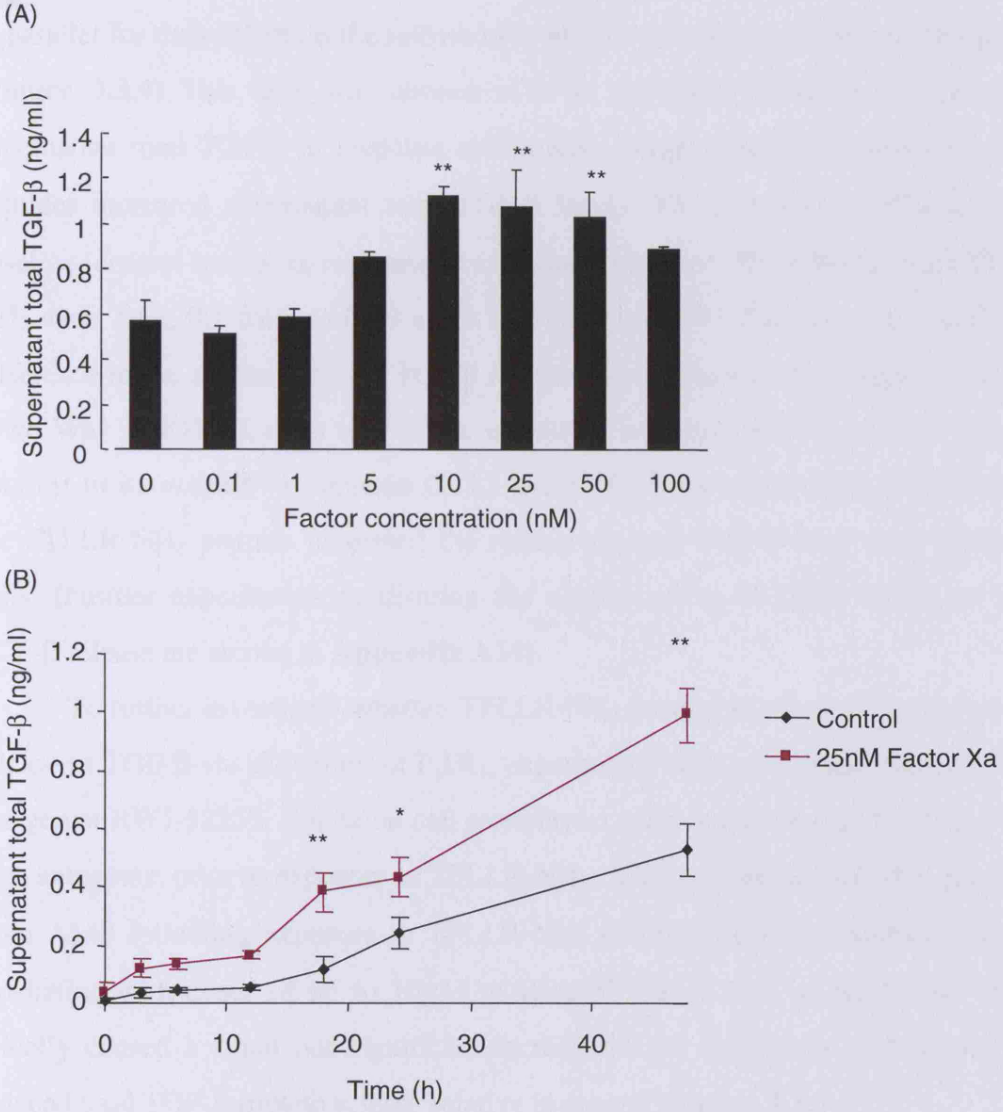


Figure 3.3.3: Factor Xa increases total TGF-β release into lung epithelial cell (A549) culture supernatants. (**A**) Factor Xa dose response on supernatant total TGF-β levels. Data represent the mean ± SEM of 3-4 replicates. **p<0.01 relative to control (One Way ANOVA with Student-Newman-Keuls post-hoc tests). (**B**) Time course of total TGF-β levels in response to media control or 25nM factor Xa. Data represent the mean ± SEM of 3-4 replicates. *p<0.05, **p<0.01 comparison with control medium at the same time-point (Two Way ANOVA with Student-Newman-Keuls post-hoc tests). All data were extrapolated from a parallel TGF-β standard curve included for each experiment.

3.3.4. Effect of the PAR_1 agonist peptide TFLLR-NH_2 on total TGF-β release from lung epithelial cells

Different concentrations of both TFLLR-NH_2 and FTLLR-NH_2 were assessed in parallel for their effect on the release of total TGF-β from A549 over an 18h period (**Figure 3.3.4**) This time was chosen as it at the point of maximal increase in supernatant total TGF-β in response to thrombin (**Figure 3.3.2**). Surprisingly, both peptides increased supernatant total TGF-β levels. The increases in TGF-β above baseline (control media) in response to exposure to 200μM TFLLR-NH_2 and FTLLR-NH_2 were 2.1 ± 0.1 fold, and 1.9 ± 0.2 fold (both $p<0.05$). There was no significant difference in the amount of total TGF-β released in response to these peptides overall (Two Way ANOVA), or at any of the individual concentrations examined. Thus in contrast to its inability to increase CCL2 and CTGF gene expression, in this setting the FTLLR-NH_2 peptide increased the release of total TGF-β from lung epithelial cells. (Further experiments confirming the agonist effect of FTLLR-NH_2 on total TGF-β release are shown in **Appendix A14**).

To further investigate whether TFLLR-NH_2 exerted its effect on epithelial cell release of TGF-β via activation of PAR_1, experiments were performed with the PAR_1 antagonist RWJ-58259. Epithelial cell monolayers were pre-incubated for 20 minutes with antagonist prior to exposure to TFLLR-NH_2. The increase in total TGF-β release from A549 following exposure to TFLLR-NH_2 (200μM) was not inhibited by pre-incubation with doses of up to 10μM of RWJ-58259. In fact, at 5μM RWJ-58259 actually caused a small but significant increase in the magnitude of TFLLR-NH_2-induced total TGF-β protein release relative to control (**Figure 3.3.5**).

Thus from these data, although it would appear that both thrombin and factor Xa are capable of causing the release of total TGF-β from lung epithelial cells (A549), there is insufficient evidence to conclude that PAR_1 activation *per se* is involved. It is possible that coagulation proteinases release TGF-β by proteolytic cleavage from the pericellular matrix; and this has been shown to be the case with mesenchymal cells by Taipale *et al* (Taipale et al. 1992). In addition it appears that TGF-β release from A549 cells may occur non-specifically in response to small peptides, since TFLLR-NH_2 and FTLLR-NH_2 appear to have near equivalent effects. Importantly this may be a phenomenon peculiar to epithelial TGF-β release since FTLLR-NH_2 has no such

effect on CCL2 protein release (**Figure 3.2A.5, Panel C**) or on CCL2 or CTGF mRNA levels (**Figure 3.2A.7** and **Figure 3.2B.3**).

Figure 3.3.4:

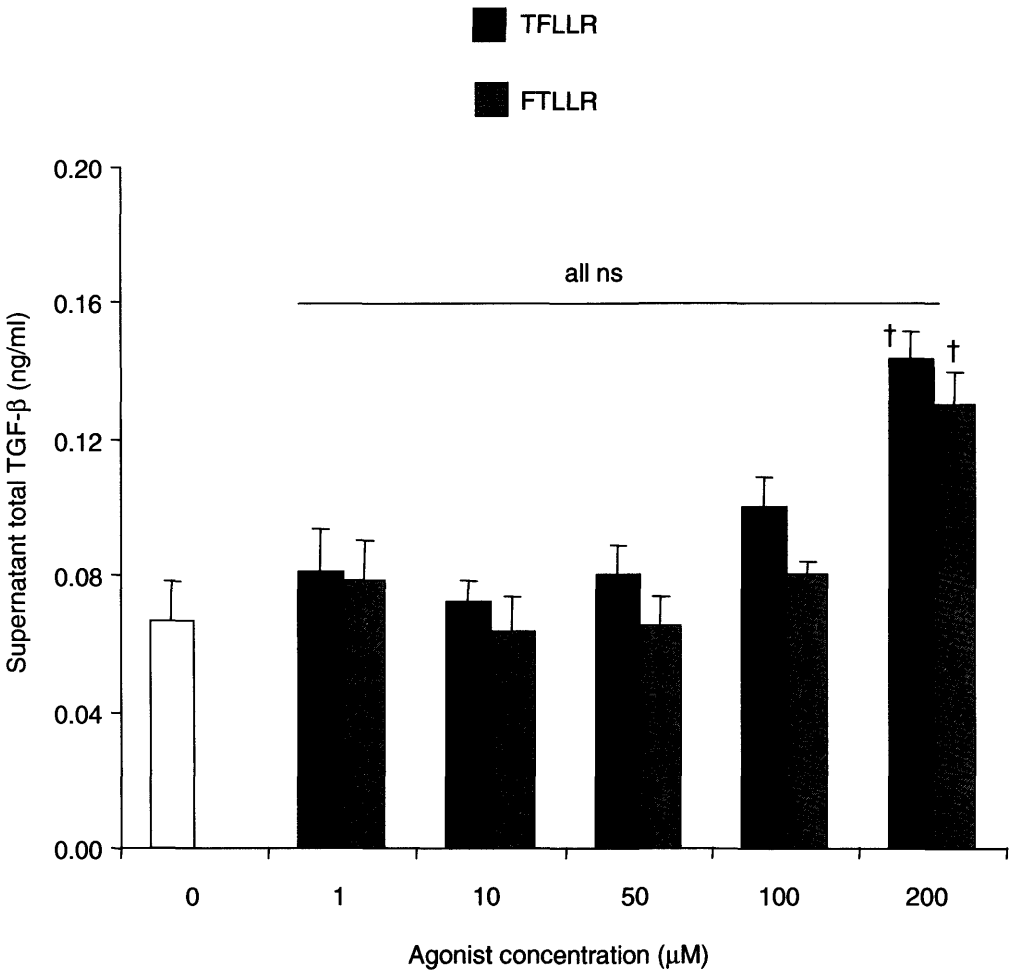


Figure 3.3.4: Effect of the PAR_1 agonist peptide TFLLR-NH_2 and the reverse control peptide FTLLR-NH_2 on supernatant total TGF-β levels. Comparison of effect of TFLLR- and FTLLR-associated total TGF-β release at increasing concentrations of peptide. Figure shows total TGF-β in supernatant for each peptide and concentration. Data represent mean ± SEM of 5 replicates. ns : not significant, overall difference between TFLLR-NH_2 and FTLLR-NH_2; †$p<0.05$ compared with media control (Two Way ANOVA with Student-Newman-Keuls post-hoc tests).

Figure 3.3.5:

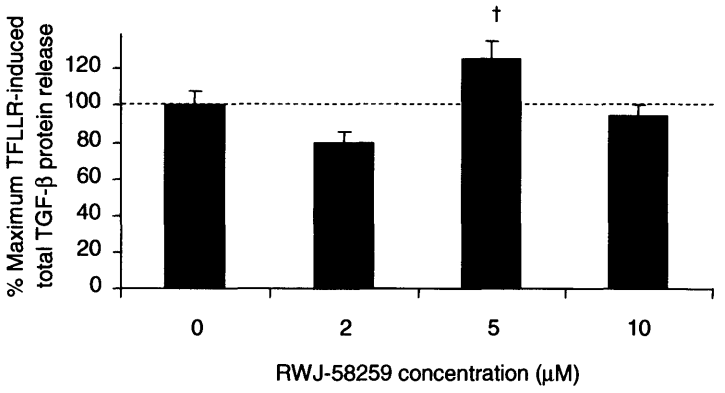


Figure 3.3.5: The selective PAR_1 antagonist RWJ-58259 does not influence TFLLR-induced total TGF-β protein release from lung epithelial cells (A549). Figure shows the effect of increasing concentrations of RWJ-58259 on total TGF-β release induced in response to 200μM TFLLR-NH_2. Data represent mean ± SEM of 4 replicates. ns comparison of TFLLR-induced TGF-β release in the presence of RWJ-58259 with TFLLR-induced TGF-β release in the absence of RWJ-58259. † $p<0.05$ compared to TFLLR-NH_2 without RWJ-58259 (One Way ANOVA with Student-Newman-Keuls post-hoc tests). Note that RWJ-58259 did not affect media control supernatant TGF-β levels.

3.3.5. Effect of thrombin and the PAR_1 agonist peptide TFLLR-NH_2 on TGF-β_1 mRNA levels in lung epithelial cells

In order to investigate whether PAR_1 agonists influenced TGF-β_1 gene expression, mouse (MLE-15) and human (A549 and BEAS-2B) lung epithelial cells were exposed to TFLLR-NH_2 (200μM) and thrombin (10nM). TGF-β_1 and 18S mRNA levels were assessed over a 12 hour time-course by real-time RT-PCR. **(Figure 3.3.6, Panel A)**.

Exposure to thrombin and TFLLR-NH_2 was associated with maximal increases in TGF-β_1 mRNA levels in A549 cells by only 1.6 ± 0.2 and 1.4 ± 0.1 fold

($p<0.05$) (**Figure 3.3.6 Panel A**); and no increases at all in BEAS-2B (**Figure 3.3.6 Panel B**) or MLE-15 cells (**Figure 3.3.6, Panel C**). (Further data supporting the minimal effect on TGF-β_1 mRNA levels in A549 is shown in **Appendix A15**).

In order to confirm the specific nature of the small but significant response observed with the PAR_1 agonist peptide TFLLR-NH_2 on TGF-β_1 mRNA levels in A549 cells, the effect of the partial reverse peptide FTLLR-NH_2 was examined in parallel. In this experiment (please see **figure 3.3.7**) a similar increase in mRNA levels in response to TFLLR-NH_2 was seen as that displayed in **Figure 3.3.6 Panel A** but the FTLLR-NH_2 peptide had no effect.

3.3.6. Effect of Factor Xa on TGFβ_1 mRNA levels in lung epithelial cells

Exposure of A549 lung epithelial cells to factor Xa had no effect on CCL2 mRNA levels but promoted a small but significant increase in CTGF mRNA levels. Factor Xa caused a dose and time-dependent increase in release of total TGF-β protein levels. To examine whether factor Xa influenced TGF-β_1 gene expression in A549, these cells were exposed to factor Xa (25nM) for up to 12h, and TGF-β_1 mRNA levels measured by real-time RT-PCR. In fact, factor Xa was found to have no significant effect on TGF-β_1 mRNA levels (**Figure 3.3.8**).

Figure 3.3.6:

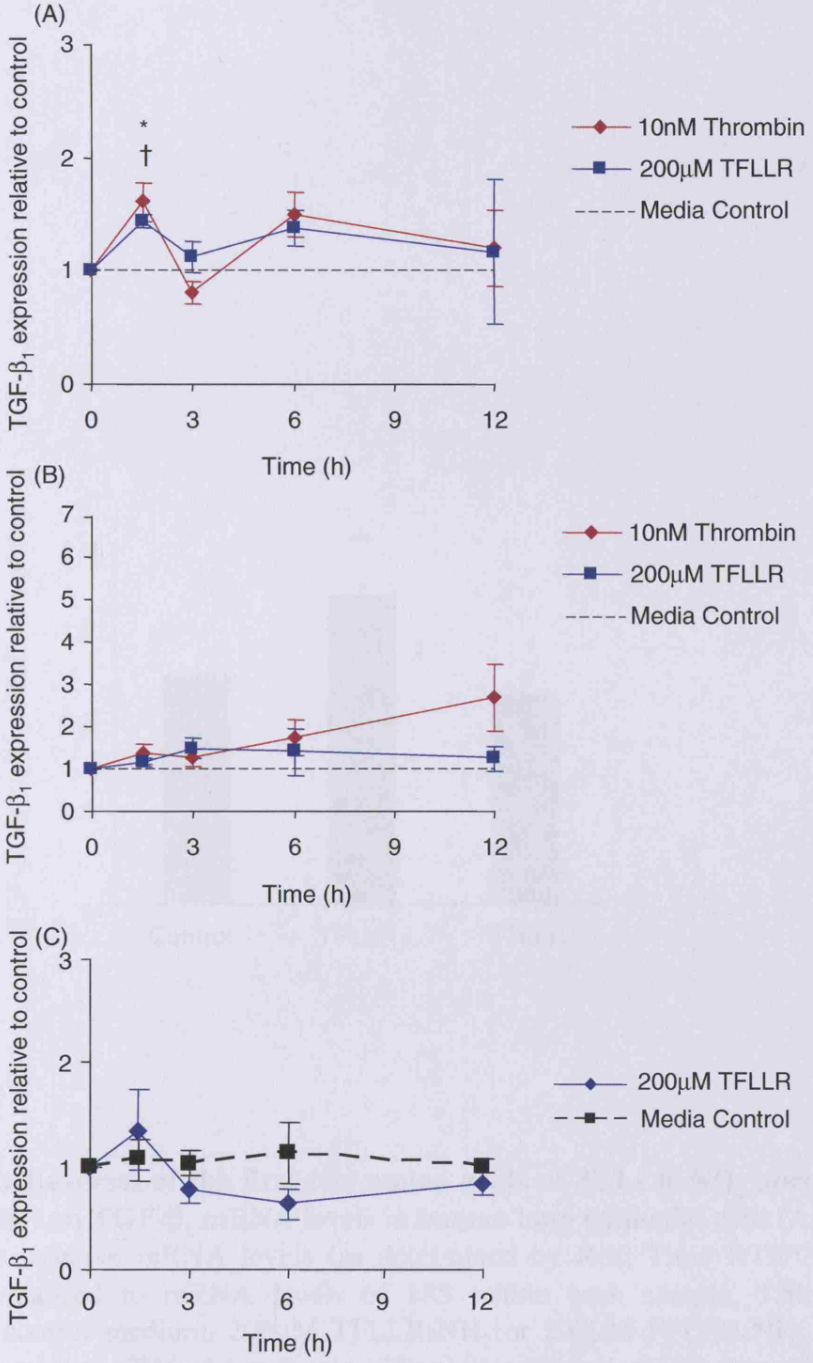


Figure 3.3.6: The PAR_1 agonist peptide TFLLR-NH_2 and thrombin have a minor effect on TGF-β_1 mRNA levels in human and murine lung epithelial cells.

Figures show mRNA levels (as determined by real Time RT-PCR) of TGF-β_1, normalised to mRNA levels of 18S within each sample, in response to 200μM TFLLR-NH_2 and/or 10nM thrombin and relative to control medium at each time-point. (**A**) A549 cells, (**B**) BEAS-2B cells, (**C**) MLE-15 cells. Data represent mean ± SEM of 3-4 replicates for each timepoint. *$p<0.05$ compared with time-point matched control (thrombin) †$p<0.05$ compared with time-point matched control (TFLLR-NH_2) (Student's t tests).

Figure 3.3.7:

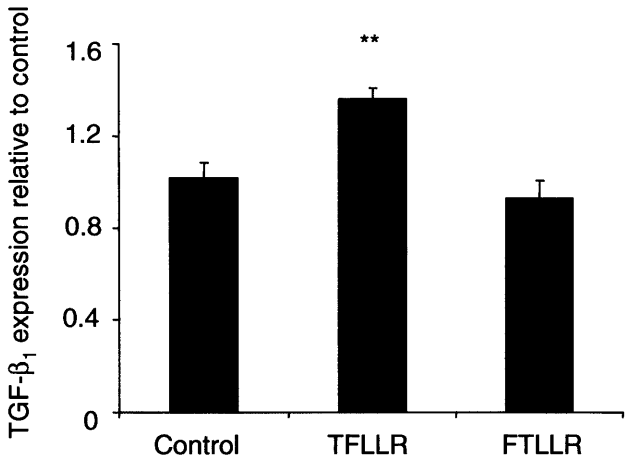


Figure 3.3.7: Reversal of the first two amino acids of TFLLR-NH_2 abrogates its minor effect on TGF-β_1 mRNA levels in human lung epithelial cells (A549)
Figures show relative mRNA levels (as determined by Real Time RT-PCR) of TGF-β_1, normalised to mRNA levels of 18S within each sample, 1.5h after exposure to control medium, 200μM TFLLR-NH_2 or 200μM FTLLR-NH_2. Data represent the mean ± SEM of 6 replicates. **$p<0.01$ comparison with control. (One Way ANOVA with Student-Newman-Keuls post-hoc tests).

Figure 3.3.8:

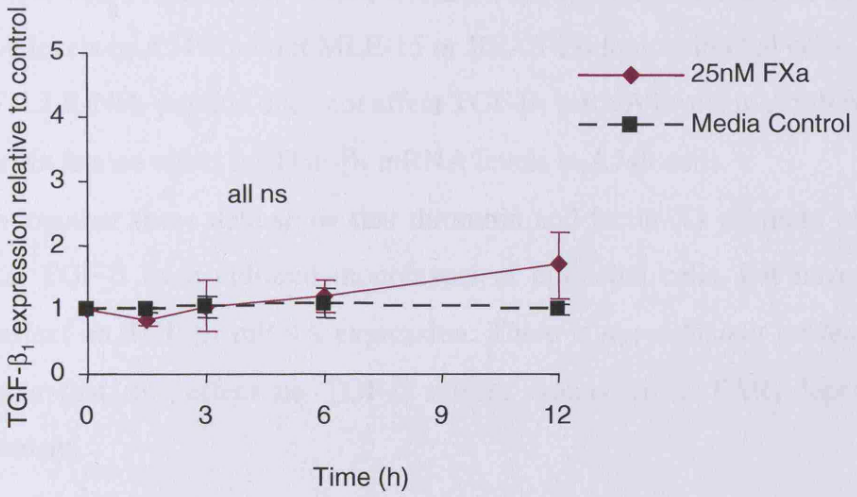


Figure 3.3.8: Factor Xa has no effect on TGF-β_1 mRNA levels in A549 cells
Figures show mRNA levels (as determined by Real Time RT-PCR) of TGF-β_1, normalised to mRNA levels of 18S within each sample, in response to 25nM Factor Xa and relative to control medium at each timepoint. ns comparison of FXa-induced mRNA levels with time-point matched control (Student's t test)

3.3.7. Summary

- Thrombin induces a concentration and time-dependent release of total TGF-β from cultured lung epithelial cells.
- Factor Xa induces a concentration and time-dependent release of total TGF-β from cultured lung epithelial cells.
- Both the TFLLR-NH_2 and the FTLLR-NH_2 partial-reverse control peptide increase release of total TGF-β from lung epithelial cells.
- The PAR_1 antagonist does not inhibit release of total TGF-β induced by TFLLR-NH_2.
- Thrombin and TFLLR-NH_2 induce a small but significant increase in TGF-β_1 mRNA levels in A549 but not MLE-15 or BEAS-2B lung epithelial cells.
- The FTLLR-NH_2 peptide does not affect TGF-β_1 mRNA levels in A549 cells.
- Factor Xa has no effect on TGF-β_1 mRNA levels in A549 cells.
- Taken together these data show that thrombin and factor Xa promote release of total TGF-β from cultured monolayers of epithelial cells, but have very little effect on TGF-β_1 mRNA expression. There is *not sufficient evidence* to conclude that this effect on TGF-β release occurs via a PAR_1-dependent mechanism.

3.4. Effect of PAR_1 deficiency on lung parenchymal architecture

3.4.1. Introduction

The main phenotypic feature that has thus far been ascribed to $PAR_1^{-/-}$ mice in the literature is a defect in endothelial vascular development which is associated with a 50% intraembryonic lethality (Griffin et al. 2001). Nonetheless, the data desribed in the first results chapter of this thesis show that $PAR_1^{-/-}$ mice fail to upregulate CCL2, CTGF, and TGF-β_1 expression in the context of bleomycin-induced lung injury. These mediators can all play important roles in the regulation of extracellular matrix protein deposition and turnover. Thus it was conceivable that chronic deficiency of these mediators might be associated with insufficient extracellular matrix protein deposition and the development of emphysema. In a similar vein, Bonniaud *et al* previously showed that mice deficient in SMAD3, a pivotal signalling intermediate of the TGF-β pathway, developed spontaneous emphysema (Bonniaud et al. 2004).

In this chapter, lung architecture was examined in aged mice to ascertain if PAR_1 deficiency was associated with the development of emphysema or other structural lung abnormality.

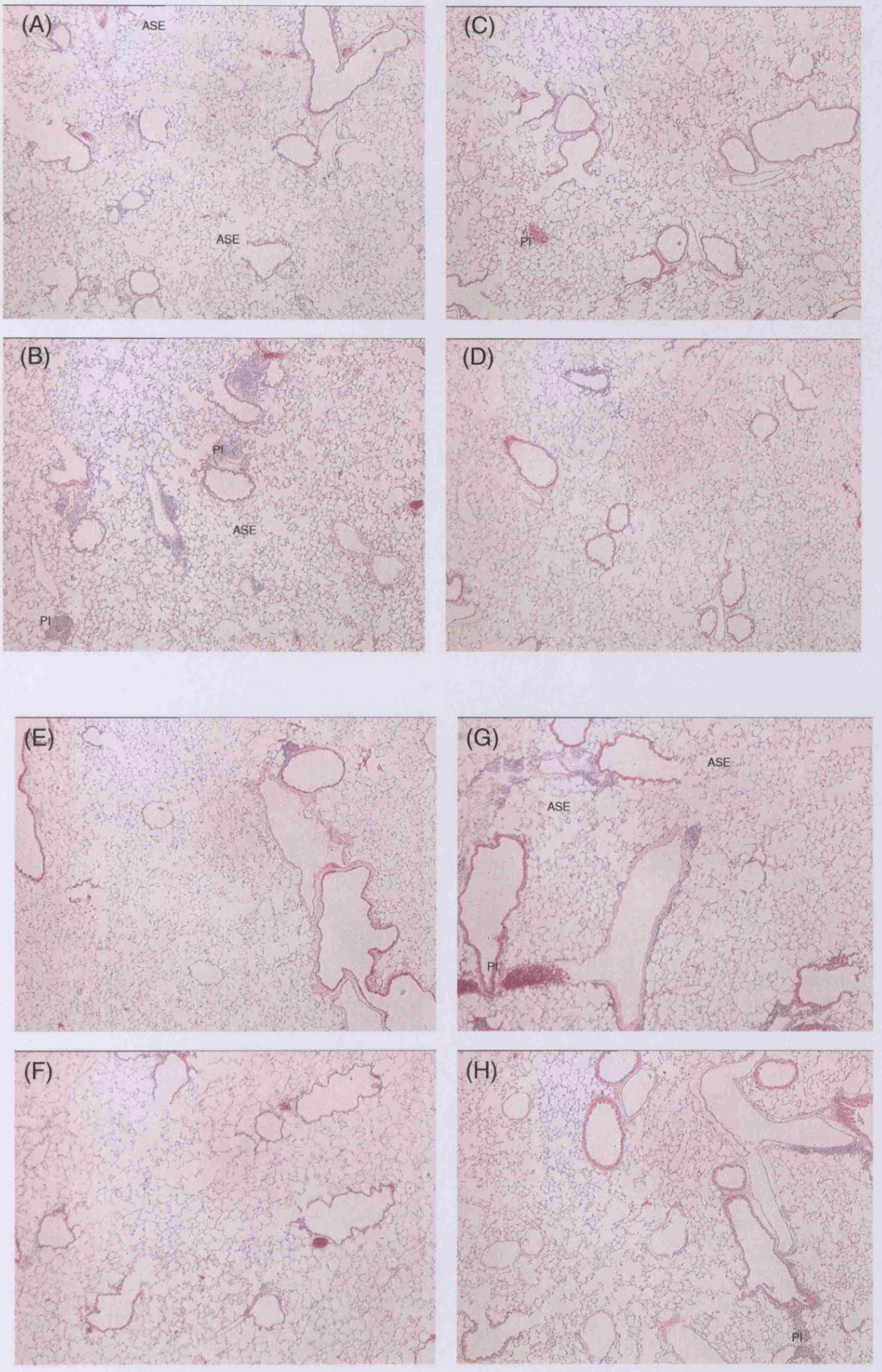
3.4.2. Effect of prolonged PAR_1 deficiency on lung architecture

In order to evaluate whether PAR_1 deficiency has any effect on the structure of the lung parenchyma over time, $PAR_1^{-/-}$ and WT mice were allowed to age for 2 years. Relative to young mice (6-8 weeks), a degree of airspace enlargement and perivascular inflammatory infiltrates were evident in sections of these lungs (**Figure 3.4.1**). However, these changes appeared equivalent for $PAR_1^{-/-}$ (**Panels E-H**) and WT mice (**Panels A-D**), suggesting that PAR_1-deficiency does not in itself accelerate the development of emphysema.

Figure 3.4.1 (please see overleaf): PAR_1 deficiency is not associated with any obvious structural lung abnormality in mice with increasing age.

Images (x 40 original magnification) of lung sections from elderly mice of between 20 and 26 months of age, stained with haematoxylin and eosin. Each picture depicts representative lung parenchyma of an individual mouse. (A-D: WT mice; E-G: $PAR_1^{-/-}$ mice). ASE = airspace enlargement. PI = pulmonary infiltrates.

Figure 3.4.1:.



3.4.3. Summary

- PAR_1 deficiency does not predispose to accelerated development of emphysema or other structural abnormality in mice with increasing age.

4. DISCUSSION

4.1 Overview

Excessive local procoagulant activity typifies fibroproliferative disorders of the lung. Coagulation proteinases such as thrombin are likely to play a major role in the pathogenesis of fibroproliferative lung disorders by the generation of potent pro-inflammatory and pro-fibrotic mediators from a number of cell types following activation of PAR_1. The dysfunctional interaction between epithelial cells and fibroblasts is increasingly recognised as an important process driving fibrogenesis in these conditions. This thesis therefore examined the hypothesis that *PAR_1 activation promotes lung injury and fibrosis via the release of a number of specific secondary mediators; with the pulmonary epithelium representing an important cellular source of these mediators.*

This thesis reports a number of novel observations. First, PAR_1-deficient mice are protected from bleomycin-induced lung inflammation and fibrosis. Second, this protection is associated with reduced lung mRNA and/or protein levels of CCL2, CTGF, and TGF-β_1. Third, PAR_1 expression is increased in the injured murine lung; and one of the sites at which this increased expression is most evident is the lung epithelium. Finally, activation of PAR_1 on mouse and human lung epithelial cells *in vitro* promotes the expression of CCL2 and CTGF, but not TGF-β_1.

Taken together the studies performed in this thesis support the notion that activation of PAR_1 in the injured lung may play an important role in the pathogenesis of fibroproliferative lung disorders by inducing local generation of pro-inflammatory and fibrotic mediators from lung epithelial cells. The following sections will discuss the results of these studies and their implications in detail.

4.2 Effect of PAR_1 deficiency on bleomycin-induced lung inflammation

Lung injury induced by intratracheal bleomycin is associated with widespread epithelial cell injury via the local generation of free radicals and reactive oxygen

species. Consequent expression of pro-inflammatory cytokines such as TNF-α, IL-1, and IL-6 by resident lung cells is likely to be important in the initiation of the cellular inflammatory response. This may occur as a direct result of cell stress or as a result of activation of a number of Toll-like receptors on the surface of these cells. Some Toll like receptors, for example Toll-like Receptor 2 have been shown to be directly activated by bleomycin (Razonable et al. 2005), while others may be activated by reactive oxygen species, proteins released from dead or dying cells, and breakdown products of extracellular matrix (reviewed in (Chaudhuri et al. 2005)).

Epithelial/endothelial barrier integrity is compromised by epithelial cell loss and results in vascular leak, which is manifest by increased protein content in bronchoalveolar lavage. Vascular leak facilitates exposure of circulating coagulation proteinase zymogens to tissue factor which is upregulated on a number of resident lung cells in both intravascular and extravascular compartments in the injured lung. This initiates the coagulation cascade which culminates in the generation of thrombin, and subsequently fibrin deposition in the pulmonary interstitium. As mentioned previously, thrombin and fibrin are each able to modulate inflammation. Fibrin may promote inflammation by serving as an important matrix for the recruitment of inflammatory cells and by influencing the release of pro-inflammatory mediators from resident cells. Thrombin may promote inflammation by activating its major cellular receptor PAR_1 on endothelial cells to promote adhesion and transmigration of circulating leucocytes, and generate potent pro-inflammatory mediators from resident lung cells.

Importantly, the attenuated responses demonstrated in $PAR_1^{-/-}$ mice in this thesis are unlikely to be due to a difference in the immediate injury caused by bleomycin between the two mouse genotypes, since BALF LDH activity at 24 hours was increased to a similar extent in WT and $PAR_1^{-/-}$ mice. BALF LDH activity is a useful indicator of lung damage (Drent et al. 1996), as it reflects cytoplasmic LDH released into the extracellular space from damaged or dead cells. At 24 hours, cell injury is likely to be caused by bleomycin-associated free radicals and reactive oxygen species, but may also potentially be aggravated by inflammation initiated by local cell injury and stress. It is not therefore surprising that the injury and inflammation is equivalent in the two genotypes at this early stage as it precedes the time-point at which significant generation of thrombin would be expected to exert any influence on inflammatory processes via the activation of PAR_1. Nevertheless this is

an important control in assessing the effect of PAR_1 on inflammation and fibrosis *in vivo*. The similar response to bleomycin in the two genotypes confirms that bleomycin produces the same initial experimental injury in $PAR_1^{-/-}$ and WT mice, and that attenuation of inflammation and fibrosis in $PAR_1^{-/-}$ mice is not attributable to altered sensitivity or response to it.

In order to examine specifically the role of PAR_1 on inflammatory processes, assessments were made of lung weight, BALF lavage protein content and BALF leukocyte numbers on day 6: a time-point at which thrombin has previously shown to be maximal in the bleomycin model (Tani et al. 1997),(Howell et al. 2001). Lung weight was expected to increase following bleomycin due to inflammatory cell infiltration, alveolar flooding, and interstitial oedema. Increases in lung weight were observed for both WT and $PAR_1^{-/-}$ mice, but the increase in $PAR_1^{-/-}$ was around half that in WT mice.

BALF leukocyte numbers were assessed as an index of lung inflammation. There was a substantial increase in BALF leukocyte number in WT but not in $PAR_1^{-/-}$ mice. In a previous comparable pilot experiment performed by Dr David Howell there was a similar trend for an attenuation in the increase of BALF leukocytes in $PAR_1^{-/-}$ mice. In this study, whilst the absolute numbers of leukocytes in response to bleomycin were different in the two genotypes, the relative proportions of polymorphonuclear leukocytes, lymphocytes, and monocyte/macrophages was unaltered. Differential counting of leukocyte subsets was therefore not performed for the purposes of the current thesis. Whilst such a reduction in BALF cell number clearly supports reduced parenchymal inflammation, it remains possible that BALF cell number may be be influenced, in part, by inflammation occurring in the airways as well as in the parenchyma. To further validate that changes observed in BALF cell number are representative of cellular infiltration of the lung parenchyma, quantitative assessment of inflammatory cell number in histological lung sections or in dispase digestions of lungs (by fluorescence activated cell sorting (FACS) could be undertaken).

There was a substantial increase in BALF protein content in both genotypes. However this response was blunted in $PAR_1^{-/-}$ mice consistent with reduced vascular leak and a lesser degree of compromise of epithelial/endothelial barrier integrity in the absence of PAR_1. Taken together the attenuations in increases in lung weight, BALF

protein content, and BALF leukocyte number in bleomycin-instilled $PAR_1^{-/-}$ mice, demonstrate a reduced inflammatory response in the absence of PAR_1.

There are a number of potential mechanisms by which PAR_1 may contribute to the inflammatory response. Activation of this receptor by either thrombin or immediate upstream coagulation proteinases on a variety of cell types from both intra- and extra-vascular compartments has been shown in *in vitro* studies to induce the production and release of a host of pro-inflammatory mediators, including amongst others IL-1β, IL-6 and IL-8 (Naldini et al. 1998),(Johnson et al. 1998),(Ueno et al. 1996),(Asokananthan et al. 2002). Activation of PAR_1 by thrombin also induces the expression of endothelial cell surface adhesion molecules, such as E- and P-selectin and intercellular adhesion molecule-1 (ICAM-1) (Sugama et al. 1992),(Shankar et al. 1994) *in vitro*. In terms of influencing BALF protein levels, both thrombin and PAR_1 peptide agonists have been shown to increase vascular permeability by both direct and nitric oxide-dependent effects on endothelial cells (reviewed in (Siflinger-Birnboim & Johnson 2003)), and via the release of histamine from mast cells (Cirino et al. 1996). Potential effects of PAR_1 activation previously described in the literature are also described in depth in **Introduction 1.4.1. Cellular effects of PAR_1 activation.** Importantly, the necessity for PAR_1 in mediating inflammatory responses *in vivo* was further confirmed in experiments where thrombin-induced increases in pulmonary microvascular permeability were shown to be abrogated in isolated lung preparations obtained from $PAR_1^{-/-}$ mice (Vogel et al. 2000).

To begin to delineate the potential mechanism by which $PAR_1^{-/-}$ mice were protected from bleomycin-induced lung inflammation, measurements were made of the pulmonary levels of two PAR_1 inducible mediators CCL2 and IL-6. CCL2 is a potent chemoattractant for mononuclear cells, and tissue levels correlate closely with infiltration of mononuclear cells in a host of human inflammatory conditions. IL-6 promotes leukocytosis and differentiation of B and T lymphocytes, and macrophages (reviewed in (Naka et al. 2002)). It also stimulates the acute phase response after tissue injury (Castell et al. 1988). This functions to limit the growth of any infective organisms, to remove harmful molecules, and to activate the repair processes to return organs to normal function. Both CCL2 and IL-6 have been implicated in the inflammatory response to bleomycin (Smith et al. 1995),(Smith et al. 1998). IL-6 levels were found to be increased to the same extent in response to bleomycin injury

in both mouse genotypes, suggesting that the upregulation of this cytokine occurs via a PAR_1-independent pathway in this model, and further that the protective response observed in $PAR_1^{-/-}$ mice cannot be explained by an attenuated IL-6 response. These findings for IL-6 levels are further in agreement with a recent study in a mouse model of sepsis, where deficiency of PAR_1 or PAR_2 alone had the same effect on IL-6 expression following endotoxic insult as observed for WT mice. However, when PAR_2 deficiency was combined with hirudin, thus additionally blocking signalling through PAR_1 and PAR_4, IL-6 levels were reduced relative to those in WT mice, suggesting that multiple PARs may be responsible for the regulation of this pro-inflammatory mediator in model of sepsis (Pawlinski et al. 2004), and possibly other models.

In contrast to IL-6, CCL2 levels were significantly reduced in bleomycin instilled $PAR_1^{-/-}$ mice compared with correspondingly injured WT mice in this study. In terms of the functional significance of this observation, anti-CCL2 antibodies have been shown to reduce leukocyte accumulation following bleomycin-induced lung injury (Smith et al. 1995), suggesting that this chemokine plays an important role in the inflammatory response to bleomycin. Although multiple PARs may mediate the cross-talk between coagulation and inflammation in certain disease settings, findings from this study suggest that PAR_1 plays a critical role in such cross-talk in this injury model.

It was initially postulated that the relative abundance of Th_1-type and Th_2-type cytokines might reveal evidence of a difference in Th_1/Th_2 cytokine profiles between $PAR_1^{-/-}$ and WT mice. Thus the Th_1 and Th_2-type cytokines: TNF-α and IL-10 levels in lung homogenates were measured.

TNF-α is a cytokine associated with Th_1 responses, and exerts pro-inflammatory effects via triggering local expression of cytokines and chemokines via NF-κB and AP-1-dependent pathways (reviewed in (Varfolomeev & Ashkenazi 2004)). TNF-α levels are increased in response to lung injury in the bleomycin model (Piguet et al. 1989),(Ortiz et al. 1998),(Gurujeyalakshmi et al. 2000),(Cavarra et al. 2004). A pro-inflammatory role for TNF-α is suggested by the observation that overexpression of TNF-α under the control of a SPC promoter *in vivo* is associated with chronic inflammation (Fujita et al. 2003), and anti-TNF-α antibodies reduce bleomycin-induced alveolitis (Piguet et al. 1989). However it seems that TNF-α may

be able to both promote and inhibit fibrosis. TNF-α promotes proliferation of fibroblasts *in vitro* (Sugarman et al. 1985), and anti-TNF-α antibodies inhibit bleomycin-induced lung fibrosis (Piguet et al. 1989). Conversely, TNF-α is able to inhibit TGF-β-induced CTGF expression and collagen synthesis by fibroblasts *in vitro* (Abraham et al. 2000), whilst TNF-α overexpression protects against murine experimental lung fibrosis (Fujita et al. 2003). Interestingly, work by Oikonomou *et al* (Oikonomou et al. 2006) suggests that the presence of TNF as a soluble form following bleomycin lung injury is a prerequisite to the development of fibrosis. TNF normally exists as both transmembrane and soluble forms, each of which is functionally active. TNF is originally produced as a 26 kDa transmembrane molecule, which must be cleaved by TNF-α converting enzyme (TACE) to release the soluble 17 kDa TNF-α. Transgenic mice expressing only the transmembrane form of TNF-α are protected from bleomycin-induced lung fibrosis, but co-administration of aerosolized recombinant soluble TNF-α with bleomycin in these mice leads to pulmonary fibrosis. In the present study, given the observation that there was a significant increase in lung TNF-α levels in response to bleomycin in WT but not in $PAR_1^{-/-}$ mice, it is tempting to speculate a pro-inflammatory and pro-fibrotic role for TNF-α following PAR_1 activation, although it is unfortunately not possible to determine whether changes in TNF-α following bleomycin in either genotype reflect changes in transmembrane or soluble fractions because TNF-α was measured in homogenates of whole lung.

IL-10 is thought to inhibit pulmonary inflammation in response to bleomycin, since IL-10-deficient mice have a higher density of inflammatory cells in the pulmonary interstitium and BALF, but no difference in the degree of fibrosis compared to WT (Kradin et al. 2004). Given that PAR_1 activation is able to promote IL-10 production in human peripheral blood mononuclear cells (Naldini et al. 2003), it was initially hypothesised IL-10 levels would be reduced in $PAR_1^{-/-}$ mice deficiency. In fact, levels of IL-10 were similar constitutively in both genotypes, and were increased by a similar amount in both genotypes following bleomycin. Thus the protective response observed in $PAR_1^{-/-}$ mice appears to be independent of IL-10. It is possible that PAR_1 activation does not induce production of IL-10 on murine lung cells as it does on human peripheral blood mononuclear cells. On a technical note, the measurable IL-10 in lung homogenates was very low; in fact slightly below the

sensitivity range for the ELISA, and it is possible, then, that real differences were missed for this reason.

4.3 Effect of PAR_1 deficiency on bleomycin-induced lung fibrosis

In addition to the observation that $PAR_1^{-/-}$ mice were protected from bleomycin-induced inflammation, a further major finding of this thesis was that $PAR_1^{-/-}$ mice were also protected from bleomycin-induced fibrosis. Assessment of the fibrotic response at day 14 revealed that total lung collagen accumulation was attenuated by about 60% in $PAR_1^{-/-}$ mice instilled with bleomycin compared with correspondingly injured WT mice. $PAR_1^{-/-}$ mice also exhibited an attenuation in the increase in lung weight and their lung architecture was visibly less disrupted by fibrotic tissue on histological examination. The observed reduction in bleomycin-induced CCL2 levels in $PAR_1^{-/-}$ mice described in this thesis may further be relevant to the reduced lung collagen accumulation in these mice. In addition to influencing inflammatory cell recruitment, there is accumulating evidence that CCL2 may influence the development of fibrosis. For example, CCL2 induces TGF-β_1 and procollagen gene expression in fibroblasts *in vitro* (Gharaee-Kermani et al. 1996), downregulates alveolar epithelial cell production of the anti-fibrotic prostanoid PGE_2 *in vitro* (Moore et al. 2003), and acts as a chemoattractant for collagen-synthesising fibrocytes *in vivo* (Moore et al. 2005). However, the most compelling evidence for the biological relevance of CCL2 in promoting fibrosis was provided by studies in which mice lacking the CC chemokine receptor CCR2 (the major CCL2 receptor) have been shown to be protected from bleomycin and fluorescein isothiocyanate (FITC)-induced pulmonary fibrosis (Gharaee-Kermani et al. 2003),(Moore et al. 2001). Of note, the reduction in lung collagen accumulation observed in CCR2-deficient mice appeared to occur without significant attenuation of the preceding inflammatory response. Moreover, studies employing anti-CCL2 gene therapy administered at early and late phases of the bleomycin response confirms that this chemokine plays an important role during the chronic fibrotic phase (Inoshima et al. 2004). In addition, given that a Th_2 cytokine profile is associated with fibrosis, it is tempting to speculate that a further possible mechanism by which CCL2 could

potentially promote fibrosis is by shifting the Th_1/Th_2 cytokine balance in favour of a Th_2 profile. Evidence to support the plausibility of CCL2 in promoting such a shift comes from studies by Karpus *et al*, in which CCL2 was shown to promote the differentiation of naïve T cells to a Th_2 phenotype *in vitro* (Karpus et al. 1997), and from studies by Gu L *et al*, in which $CCL2^{-/-}$ mice on a Balb/c background were unable to mount the Th_2 response to *Leishmania major* typical of Balb/c mice (Gu et al. 2000).

The expression of two other PAR_1-inducible pro-fibrotic mediators, TGF-β_1 and CTGF, was examined in $PAR_1^{-/-}$ mice relative to WT mice. TGF-β_1 and CTGF immunoreactivity was significantly reduced in bleomycin-instilled $PAR_1^{-/-}$ mice relative to bleomycin-instilled WT mice. Immunoreactivity in these studies reflects both positive staining from resident lung cells, as well as from locally activated cells, and recruited inflammatory cells. However, a difference in immunoreactivity between lungs of bleomycin-treated WT and $PAR_1^{-/-}$ mice was apparent for both the interstitium and the alveolar wall, independently of whether there was evidence of increased cell number or matrix accumulation. This suggests that there may be a difference in local upregulation of CTGF and TGF-β_1 between the two mouse genotypes. Corresponding changes at the mRNA level for CTGF further support this notion, although additional studies (e.g. *in situ* hybridisation) might be useful to validate the observation, particularly in the case of TGF-β.

The changes in immunoreactivity were also preceded by a significant reduction in CTGF mRNA levels on day 7. This observation is in agreement with a previous report that direct thrombin inhibition attenuates bleomycin-induced lung collagen accumulation and CTGF mRNA levels (Howell et al. 2001) and further suggests an important role for thrombin as a major PAR_1 activator in this model. In contrast, although TGFβ_1 protein expression in the injured lung was reduced by over half in bleomycin-instilled $PAR_1^{-/-}$ mice compared with correspondingly injured WT mice at day 14, there was no detectable change in TGFβ_1 mRNA expression at 7 or 14 days. Although not a universal finding, this lack of an effect at the mRNA level has previously been reported by others. For example, Kaminski *et al* were unable to detect increases in TGF-β_1, -β_2 and -β_3 mRNA in this model, even though the signature gene expression profile associated with increased TGF-β_1 signaling was clearly evident (Kaminski et al. 2000).

The observation that PAR_1 deficiency is associated with reduced TGF-β_1 immunoreactivity in response to bleomycin injury is particularly of interest given the compelling evidence that TGF-β is a major pro-fibrotic mediator in this model (Khalil et al. 1989), as well as in patients with fibrotic lung disease (Khalil et al. 1991),(Khalil et al. 1996), and suggests that this may represent an important pathway by which PAR_1 activation contributes to the fibrotic phase in this model. It is important to point out that PAR_1 is also associated with reduced immunoreactivity for CTGF and TGF-β_1 in saline control mice. Thus it is possible that PAR_1 activation also influences levels of these growth factors in the normal uninjured lung, although in this study there was no evidence of any structural differences within the lung parenchyma of uninjured aged $PAR_1^{-/-}$ relative to WT mice.

It is likely that the differences in TGF-β_1 and CTGF immunostaining between WT and $PAR_1^{-/-}$ mice are due to PAR_1 activation only increasing expression of these proteins in WT mice. Nevertheless, there may be additional or alternative reasons for these differences in immunostaining. Firstly, these growth factors could bind to a relatively more abundant extracellular matrix in WT mice. In addition to inducing procollagen expression (Chambers et al. 1998), PAR_1 activation also increases production of proteoglycans (Cizmeci-Smith & Carey 1997) and fibronectin (Armstrong et al. 1996). Active TGF-β_1 can bind directly to proteoglycans, fibrillin, fibronectin and type IV collagen (Taipale et al. 1992), although the majority of matrix-associated TGF-β_1 is in the latent form localized to this site via binding of LTBP to the matrix (Taipale et al. 1994). CTGF also readily localizes to the extracellular matrix (Kireeva et al. 1997) via its ability to bind to heparan sulphate proteoglycans (Gao & Brigstock 2003). It is possible then that a difference in the extent of CTGF and TGF-β_1 localization to the extracellular matrix between the two genotypes contributes to the differences in immunostaining observed. Secondly, whilst there is good evidence that PAR_1 activation on a number of cell types influences the production of pro-fibrotic mediators, it is not possible to rule out the possibility that protection from bleomycin-induced fibrosis in $PAR_1^{-/-}$ mice results, at least in part, from an attenuated early inflammatory response in this model, although there is now increasing evidence that inflammation is in fact not an essential precursor or requirement for fibrogenesis (Huaux et al. 1998),(Munger et al. 1999),(Adamson et al. 1988).

4.4 Effect of PAR_1 deficiency on fibrin formation

Importantly, assessment of the extent of fibrin deposited at day 6, which corresponds temporally with maximal thrombin generation following bleomycin administration (Tani et al. 1997),(Howell et al. 2001), revealed apparently similar increases for both genotypes suggesting that these attenuated responses were not due to differences in fibrin formation between the two mouse genotypes. In addition, it strongly supports the notion that the observed differences in inflammatory and fibrotic responses are due to coagulation proteinases such as thrombin activating PAR_1 rather than promoting coagulation. Hence whilst fibrin has the potential to influence inflammation by promoting the release of pro-inflammatory mediators and serving as a provisional matrix on which inflammatory cells can adhere, these effects may not be relevant to the difference in inflammatory response observed between $PAR_1^{-/-}$ and WT mice.

In addition to modulating inflammation, fibrin has also been implicated in promoting fibrosis *in vivo*. In support of the hypothesis that fibrin may contribute to the progression of fibrosis, experimental strategies that would be expected to favour fibrin clearance, such as deletion of the PAI-1 gene (Hattori et al. 2000) or overexpression of uPA (Sisson et al. 1999), have been associated with an attenuation in fibrosis following bleomycin instillation. However, it has recently become apparent that such experimental manipulation of the plasminogen system may exert antifibrotic effects by increasing local levels of the anti-fibrotic mediator hepatocyte growth factor (HGF), rather than reducing fibrin deposition (Hattori et al. 2004). In addition, since fibrinogen knockout mice are not protected from bleomycin-induced lung fibrosis (Hattori et al. 2000),(Ploplis et al. 2000) there is not insufficient evidence to conclude that fibrin exerts a direct pro-fibrotic influence *in vivo*. In the present study, the similar extent of fibrin deposition in both WT and $PAR_1^{-/-}$ mice on day 6 is consistent with fibrin not being relevant to the attenuated fibrotic response in $PAR_1^{-/-}$ mice.

4.5 PAR_1 expression is increased in response to lung injury

PAR_1 immunostaining was markedly increased in wild-type mice in response to bleomycin injury, and this was most apparent on epithelial cells and macrophages, although abundant spindle shaped fibroblast-like cells also stained prominently for PAR_1 in fibrotic foci. In our laboratory increased PAR_1 mRNA levels following bleomycin injury have also been demonstrated in homogenates of rat lung by northern blotting (Dr David Howell PhD thesis 2003) and in homogenates of mouse lung by real-time RT-PCR (personal communication and unpublished data, Dr Chris J Scotton, Centre for Respiratory Research UCL,). In support of injury-dependent local upregulation of PAR_1 expression, real-time RT-PCR in the current study demonstrated around a 5-fold increase in PAR_1 mRNA in lavaged cells, which were predominantly macrophages, 14 days after bleomycin. However it is of course possible that this reflects a change in the relative proportions of PAR_1-expressing inflammatory cells rather than increased PAR_1 expression *per se*. In order to confirm that the increased PAR_1 immunostaining on injured lung epithelial cells was attributable to increased gene expression, *in-situ* hydridisation studies would be useful. Alternatively, laser capture microdissection could be employed to isolate epithelial cells for real-time RT-PCR from uninjured and injured lung.

There are a number of potential mechanisms by which PAR_1 expression may be elevated in the injured lung. Expression of this receptor is modulated by a variety of mediators and stimuli relevant to tissue injury. PAR_1 expression has been shown to be increased in response to thrombin in endothelial cells (Ellis et al. 1999), to TGF-β_1 in vascular smooth muscle cells (Schini-Kerth et al. 1997), and to IL-1β, TNF-α and TGF-β_1 in skeletal muscle (Mbebi et al. 2001). Furthermore, monocyte differentiation has been shown to correlate with increased PAR_1 expression *in vitro* (Naldini et al. 1998). Importantly, activation of PAR_1 by thrombin induces fibroblast to myofibroblast differentiation (Bogatkevich et al. 2001) and PAR_1 expression by these cells has also been shown to be increased *in vitro* and *in vivo* (D'Andrea et al. 2001).

Experiments in this thesis strongly suggest that PAR_1 expression increases in the injured lung. It is plausible that such increased PAR_1 expression is important in the exaggerated production of PAR_1-dependent mediators following lung injury.

Whether this is indeed the case, and the precise mechanisms by which PAR_1 expression is regulated are important questions to address in further work.

4.6 Effect of lung epithelial cell PAR_1 activation on CCL2 and CTGF expression

PAR_1 activation in the injured lung is likely to influence inflammation and fibrosis by a number of mechanisms which involve a range of different cell types and secondary mediators. Since protection in PAR_1-deficient mice is associated with reduced lung levels of CCL2, CTGF, and TGF-β_1, it follows that these PAR_1-dependent mediators may promote inflammation and/or fibrosis following PAR_1 activation in the injured lung.

In light of the prominent PAR_1 immunostaining on epithelial cells in the injured murine lung and the growing body of evidence implicating epithelial cells as being an important source of inflammatory and fibrotic mediators, the effect of PAR_1 activation on production of the secondary mediators CCL2, CTGF, and TGF-β_1 by lung epithelial cells was examined. In this thesis, an attempt was made to comprehensively evaluate the effect of PAR_1 activation on generation of the described secondary mediators from lung epithelial cells *in vitro*. Possible *in vivo* experiments to support the results of these *in vitro* studies are outlined in a later section: **4.10 Further work.**

In the first instance, real-time RT-PCR was employed to assess whether the lung epithelial cells used for *in vitro* experiments express PAR_1 under the culture conditions in which the experiments were planned. Mouse lung fibroblasts and human foetal lung fibroblasts were used as positive controls as they are known to express PAR_1 and be responsive to PAR_1 activation from previous studies performed at the Centre for Respiratory Research (Chambers et al. 1998),(Chambers et al. 2000). In this study, all lung epithelial cell lines examined (MLE-15, A549, BEAS-2B) and primary human lung alveolar epithelial cells were found to express PAR_1 *in vitro*.

Both A549 and primary alveolar epithelial cells produce CCL2 constitutively as has been described previously (Pechkovsky et al. 2005). Thrombin and the PAR_1 agonist peptide TFLLR-NH_2 significantly increased CCL2 protein release from both

A549 and primary human lung alveolar epithelial cells, although the relative increase in CCL2 protein release over baseline was lower in primary cells. Taken together with the observation that both PAR_1 and CCL2 immunostaining localise to epithelial cells in adjacent areas of injured lung, this strongly suggests that lung epithelial cells may be a biologically relevant potential source of CCL2 following PAR_1 activation *in vivo*.

It is possible that the reduced magnitude of PAR_1 response in primary cells is attributable to a lower expression of PAR_1 relative to A549 (**figure 3.2A.14**). However these primary cells may be more representative of alveolar epithelial cells in uninjured lung. Given the observation that epithelial PAR_1 immunostaining increases in response to lung injury, it is tempting to speculate that hyperplastic alveolar epithelial cells *in situ* in fibroproliferative lung diseases reflect injured and/or activated cells and similarly express more PAR_1, and consequently exhibit PAR_1-dependent responses of a greater magnitude. In support of this notion, the injury related factors IL-1β, TNF-α and TGF-β_1 have previously been shown to increase PAR_1 expression on myotubes, which then also display a marked increase in the calcium signal obtained in response to thrombin (Mbebi et al. 2001).

In vitro experiments in this thesis also validate lung epithelial cells as a potential source of CTGF following PAR_1 activation. Thrombin increased CTGF protein production in A549 cells as demonstrated by western blotting and immunocytofluorescence studies. Interestingly, in initial attempts at western blotting of A549 epithelial cell lysates, CTGF was not detected in a cell layer lysed with RIPA buffer or in the culture supernatant. However, it was detected using western blotting when the cell layer and matrix were lysed in 8M Urea. This is consistent with CTGF being tightly (covalently) associated with the pericellular matrix following secretion, and not released in substantial amounts into the cell culture supernatant. Of additional interest was the observation from immunocytofluorescence studies that CTGF only appeared to be associated with a subpopulation of cells following their exposure to thrombin. Distinct subpopluations of A459 cells in culture have been described previously (Croce et al. 1999), and it is possible that different subpopulations express differing amounts of various proteins, including CTGF. Alternatively it is possible that only cells which are in a certain phase of the cell cycle express CTGF. Consistent with this notion, a previous study has shown that type II alveolar epithelial cells which appeared to be proliferating were immunoreactive for CTGF in IPF lung sections (Pan et al. 2001). Finally it is possible that this observation could be explained by a

processing artefact whereby pericellular matrix-associated CTGF is lost from the vicinity of a proportion of the cells stained, although the propensity for CTGF to tightly associate with pericellular matrix components suggests this is less likely.

Real-time RT-PCR studies showed that expression at the mRNA level of CCL2 and CTGF in response to PAR_1 agonists was closely mirrored at the protein level. Exposure of human and mouse epithelial cells to thrombin or TFLLR-NH_2 promoted significant increases in CCL2 and CTGF mRNA levels. The smaller increases in CCL2 and CTGF mRNA levels in response to TFLLR-NH_2 in mouse than in human lung epithelial cells is interesting. Given that the magnitude of certain cellular responses in human epithelial cells correlated with relative PAR_1 expression (**please see fig 3.2A.14**), it is possible that a reduced response in mouse epithelial cells is attributable to reduced PAR_1 expression compared to human lung epithelial cells *in vitro*. Alternatively, the small magnitude of response in murine cells to the human TFLLR-NH_2 PAR_1 agonist peptide could be explained by a species difference. Whilst TFLLR-NH_2 has been shown to activate PAR_1 on murine cells (Fang et al. 2003), its amino acid sequence is closer to the human SFLLR than to the mouse SFFLR tethered ligand sequence. Hence it is possible that it is therefore less effective at activating murine PAR_1. Alternatively differences in response in MLE-15 cells might relate to the culture conditions and supplements in which they are maintained. In order to retain their type II alveolar epithelial phenotype, MLE-15 cells are cultured in a medium (HITES) supplemented with a number of hormones and growth factors. Although these cells were quiesced in a serum- and supplement-free medium for 24 hours before exposure to TFLLR-NH_2, a number of the supplements previously in the culture medium up until that point have been shown to influence expression of a variety of genes including CCL2 and CTGF. Supplements possibly relevant in this regard are glucocorticoids (Natori et al. 1997),(Matsuda et al. 2005),(Rageh et al. 2001), insulin (Mamounas et al. 1991), albumin and transferrin (Wang et al. 1997), and estradiol (Xie et al. 2002). It is possible then that these supplements interfered with the regulation of CCL2 and CTGF by PAR_1.

The difference in the magnitude of the response obtained for A549 and BEAS-2B is interesting. As alluded to previously, it is possible that a greater increase in CCL2 and CTGF mRNA levels in BEAS-2B cells might be related to the high expression levels of PAR_1 (**figure 3.2A.14**) in these cells compared with A549 cells.

The potential of airway epithelial cells to produce pro-inflammatory and pro-fibrotic mediators *in vitro* is also worthy of note. These observations are consistent with the notion that these cell types may represent a source of pro-inflammatory and pro-fibrotic mediators, in addition to hyperplastic type II alveolar epithelial cells, in fibroproliferative lung disorders.

Factor Xa is able to activate PAR_1 (Riewald et al. 2001) as well as PAR_2 (Bono et al. 2000) depending on cell type and co-factor expression. However, in contrast to thrombin, Factor Xa did not significantly influence CCL2 mRNA levels in A549 cells, and increased CTGF mRNA levels by less than half that observed in response to thrombin. This may reflect functional selectivity of PAR_1, whereby different receptor agonists are coupled to distinct intracellular signalling pathways and subsequently mediate different cellular responses following PAR cleavage (Riewald & Ruf 2005),(McLaughlin et al. 2005b). Alternatively, it is possible that this reflects a lower affinity of factor Xa than thrombin for PAR_1. Higher concentrations of factor Xa than the 25nM used in this thesis are probably not physiologically relevant *in vivo*, since the plasma concentration of the zymogen factor X is around 170nM (Halkier.T 1991), and the amount of factor Xa in BALF of injured rat lung has been reported to be in the region of 1.4nM (Chan et al. 2006). Coversely, slightly higher concentrations of thrombin than FXa may be physiologically relevant *in vivo*. The circulating concentration of prothrombin is around 1000nM, but a physiologically relevant concentration (in the lung) of thrombin is probably around 10nM-25nM, based on the observation of BALF concentrations of around 6nM in healthy volunteers, and 13nM in systemic sclerosis patients (Hernandez-Rodriguez et al. 1995). In addition, factor Xa by itself has been shown to have a lower affinity for PAR_1 than when present as a complex associated with tissue factor and factor VIIa (Riewald et al. 2001). Thus it is possible that factor Xa is less biologically important than thrombin in the generation of PAR_1-dependent mediators described in this thesis.

In order to determine whether thrombin's influence on CCL2 and CTGF mRNA levels was via PAR_1, the specific PAR_1 antagonist RWJ-58259 was utilized. This is a small indole-based compound which specifically inhibits the tethered ligand of PAR_1 binding to the second extracellular loop, and thus inhibits receptor activation. RWJ-58259 inhibited PAR_1 responses when used at concentrations between 2 and 6μM. Other investigators have used similar concentrations and found the IC_{50} of

RWJ-58259 to be in the μM range (Damiano et al. 2003),(Andrade-Gordon et al. 2001). Thrombin-induced CCL2 mRNA levels were totally abrogated in A549 cells, and were substantially attenuated in BEAS-2B cells. Similarly thrombin-induced CTGF mRNA levels were substantially attenuated in A549 and BEAS-2B cells. The reduced inhibitory effect of the antagonist on responses in BEAS-2B cells is interesting and may have several explanations. First, it is possible that thrombin additionally influences CCL2 and CTGF mRNA via other receptors in these cells. These could include other PARs or the so-called non-proteolytically activated receptor (n-PAR). This hypothetical receptor is presumed to be mediate cellular effects of thrombin that are independent of proteolysis (Jenkins et al. 1995). In order to address this possibility, the effect of proteolytic inhibitors on abrogating the effect of thrombin on CCL2 and CTGF mRNA levels in BEAS-2B cells would be a useful further experiment. Second, it is possible that cleaved PAR_1 is able to transactivate PAR_2 in these cells, as has been demonstrated in endothelial cells (O'Brien et al. 2000). As RWJ-58259 blocks the interaction of the tethered ligand to the second extracellular loop of the PAR_1 receptor, it would not prevent PAR_2 transactivation. However, the substantial attenuation of thrombin-induced CCL2 and CTGF mRNA levels in A549 and BEAS-2B cells with RWJ-58259 suggests that thrombin is acting largely, if not wholly, via PAR_1 in these cells. Nevertheless, two useful further experiments would be to assess: i) whether a higher concentration of RWJ-58259 completely abrogated these responses, and ii) the effect of the PAR_2 agonist SLIGRL on CCL2 and CTGF mRNA levels.

In addition to being able to activate PAR_1, thrombin can activate PAR_3 and PAR_4, although PAR_3 has not been shown to signal directly. However, the AYPGKF peptide, which activates PAR_4 on human platelets, was found to have no influence on CCL2 mRNA levels in A549 cells. In addition real-time RT-PCR demonstrated that PAR_4 mRNA was not expressed by these cells. This is in agreement with previous work by Grishina *et al* showing PAR_4 to be absent on A549 cells (Grishina et al. 2005), although contrasts with work by Asokananthan *et al*, which showed PAR_4 to be present (Asokananthan et al. 2002). Taken together the data obtained in experiments from the current thesis show that thrombin-induced CCL2 and CTGF expression in lung epithelial cells is PAR_1-dependent and PAR_4-independent.

Increases in CCL2 mRNA and protein levels in response to TFLLR-NH_2 were unaffected by pertussis toxin but attenuated in a concentration-dependent manner by

the protein kinase C inhibitor Ro-31-8425. Hence these responses are pertussis-independent but protein-kinase C dependent. Of note, it is possible that following PAR_1 activation, CCL2 protein release is regulated by a pathway which is, *in part*, protein kinase C-independent. In support of this notion, in response to TFLLR-NH_2 and an equivalent concentration of Ro-31-8425, CCL2 protein release was inhibited to a lesser extent than CCL2 mRNA levels.

CTGF mRNA levels were similarly unaffected by pertussis toxin, but inhibited in a dose-dependent manner by Ro-31-8425, consistent with CTGF also being expressed in response to PAR_1 activation via a protein kinase C-dependent pathway. Interestingly, the extent of inhibition of TFLLR-induced CTGF mRNA levels by Ro-31-8425 was less than that of TFLLR-induced CCL2 levels. This suggests that PAR_1-induced CTGF expression could be additionally mediated by other non protein-kinase C pathways and possibly also downstream of coupling to other G-protein subunits. It is tempting to speculate a role for a $G\alpha_{12/13}$-dependent pathway since one of its putative downstream mediators Rho A has been shown to be essential for upregulation of CTGF expression by 5HT, lypophosphatidic acid (LPA) and TGF-β_1 in mesangial cells (Hahn et al. 2000), and simvastatin in fibroblasts (Eberlein et al. 2001). Moreover overexpression of constitutively active Rho A induces CTGF expression in fibroblasts (Ott et al. 2003).

Taken together these data are consistent with PAR_1 activation promoting epithelial CCL2 and CTGF expression, at least in part, via coupling to $G\alpha_q$. Further studies would clearly be needed to confirm this, and delineate the precise signalling pathways involved, although this would have been beyond the scope of this thesis. Further studies would also be useful to establish whether CCL2 or CTGF expressed in lung epithelial cells possesses any biological functional activity. Unfortunately, results of experiments examining whether CCL2, derived form epithelial cells following PAR_1 activation, possessed biological functional activity were inconsistent. Therefore it is not possible to conclude whether or not CCL2 derived in this way possesses biological functional activity. In the first instance, future studies could be directed toward further optimisation of monouclear cell chemotaxis assays in order to establish whether epithelial cell-derived CCL2 promotes mononuclear cell chemotaxis. Alternatively, similar assays could be designed to examine whether epithelial-derived

CCL2 promotes chemotaxis of fibrocytes, or downregulates production of PGE_2 by other alveolar epithelial cells.

4.7 Effect of PAR_1 activation on lung epithelial cells on TGF-β expression and release

To investigate whether PAR_1 activation influences TGF-β release from lung epithelial cells, A549 were exposed to thrombin and factor Xa. Both these proteinases increased the release of total TGF-β into the cell culture supernatant in a concentration and time-dependent manner. There was a large increase in the amount of total TGF-β in culture supernatants at concentrations of 100nM of thrombin compared with 10, 25, and 50nM, which were equally efficacious in their ability to influence TGF-β release. The distinct effect at this high dose could a consequence thrombin being toxic to cells, promoting cell necrosis and/or apoptosis. Both mechanical cell damage (Howat et al. 2002) and apoptosis (Hodge et al. 2002) have previously been shown to increase TGF-β release from lung epithelial cells. Whilst cell toxicity was not obviously apparent from visual inspection of cell monolayers by light microscopy in the current study, assessment of supernatant LDH levels would be a useful further experiment to investigate this possibility.

The PAR_1 agonist peptide TFLLR-NH_2 also increased supernatant total TGF-β levels. 200μM TFLLR-NH_2 increased total TGF-β release by around two-fold. However a similar nearly two-fold increase was also obtained with the partial reverse control peptide FTLLR-NH_2. The effect of TFLLR-NH_2 was not significantly different from that of FTLLR-NH_2 overall or at any dose examined. In contrast the FTLLR-NH_2 peptide did not display such an agonist effect in terms of influencing CCL2 and CTGF mRNA levels, or CCL2 protein levels. Hence this is consistent with TFLLR-NH_2 and FTLLR-NH_2 influencing TGF-β release from epithelial cells via a PAR_1-independent mechanism. Possible mechanisms of this TGF-β release could be in response to these peptides activating an alternative cellular receptor other than PAR_1, or as a non-specific response to exposure to small peptides. The observation that it was not possible to antagonize TFLLR-induced TGF-β release with

concentrations of PAR_1 antagonist up to 10μM further led to the conclusion that TFLLR-NH_2 was not acting via PAR_1 in this setting.

In light of the above findings with regard to the TFLLR-NH_2 peptide, it is likely that the influence of thrombin and factor Xa on TGF-β release are also independent of PAR_1 activation, although assessment of whether these responses failed to be attenuated by the PAR_1 antagonist would clearly be required to validate this. It is possible that the effects of these proteinases occur via proteolytic cleavage and release of TGF-β from the pericellular matrix, but such a proteolytic effect would obviously not explain the release of TGF-β in response to TFLLR-NH_2 and FTLLR-NH_2. Such a proteolytic effect has been previously demonstrated in cultured mesenchymal cells in response to plasmin and thrombin (Taipale et al. 1992). In these studies these proteinases caused a decrease of matrix-associated TGF-β with a concurrent increase TGF-β in the supernatant. However for thrombin, this effect was not readily apparent with thrombin concentrations below 5units/ml (around 125nM), which is much higher than the concentration used in the experiments performed during this thesis. In view of insufficient evidence to conclude that thrombin/ factor Xa and TFLLR-induced TGF-β protein release was PAR_1 mediated, the mechanism of TGF-β release from lung epithelial cells *in vitro* was not pursued further in experiments of this thesis.

Real-time RT-PCR experiments were performed to establish whether PAR_1 activation influenced TGF-β1 mRNA levels in lung epithelial cells. There was a small significant increase in TGF-β_1 mRNA levels in response to thrombin and TFLLR-NH_2 in A549 cells, but not in other cell types examined. The biological significance of this small ~1.5 fold increase is debatable. The observation that in contrast to CTGF, TGF-β_1 mRNA levels were not found to be differentially increased at day 7 in $PAR_1^{-/-}$ mice further supports the notion that PAR_1 activation does not significantly influence TGF-β_1 at the transcriptional level.

Release of supernatant TGF-β in response to thrombin is unlikely to be dependent on TGF-β_1 expression, since cellular mRNA levels are not substantially affected by thrombin. It would, in theory, be possible for thrombin to increase TGF-β expression and release by an effect on translation but not transcription. In order to determine whether thrombin-induced TGF-β release is independent of TGF-β

expression, experimental approaches using actinomycin-D, cyclohexidine, and brefeldin-A would be useful. Actinomycin-D: an inhibitor of transcription, and cycloheximide: an inhibitor of protein synthesis, could be used to investigate whether the response is dependent on TGF-β transcription and *de novo* TGF-β protein production. Brefeldin A, an inhibitor of protein transport and exocytosis could be used to investigate whether thrombin-induced TGF-β release was via a mechanism involving exocytosis of pre-formed TGF-β stored within from intracellular storage vesicles.

TGF-β bioactivity is regulated on a number of levels including transcription, translation, storage and release, localization, and activation. It is conceivable that PAR_1 could promote TGF-β activation. This latter possibility was not specifically addressed in the experiments outlined in this thesis, although interestingly, during the course of experiments performed in this thesis, PAR_1 activation has been shown to induce expression of the latent TGF-β activator thrombospondin-1 in endothelial cells (McLaughlin et al. 2005a), and to promote TGF-β activation in lung epithelial cells via a $\alpha_v\beta_6$ integrin-dependent mechanism (Jenkins et al. 2006). However, the *in vivo* experiments of this thesis demonstrated a reduction in *total* TGF-β_1 immunostaining in $PAR_1^{-/-}$ mice. Possible explanations for this are reduced specific extracellular matrix components to which TGF-β_1 can bind in $PAR_1^{-/-}$ mice, or that PAR_1 activation may increase TGF-β_1 expression/release in resident lung cells other than epithelial cells. Alternatively, PAR_1 activation may *indirectly* increase lung epithelial cell TGF-β expression/release by a mechanism dependent on the expression of other mediators.

4.8 Characterization of lung parenchymal architecture in $PAR_1^{-/-}$ mice

The results oulined in this thesis demonstrate that $PAR_1^{-/-}$ mice are protected from bleomycin-induced lung inflammation and fibrosis. Other published reports have shown that $PAR_1^{-/-}$ mice are also protected from experimentally induced arthritis (Yang et al. 2005), glomerulonephritis (Cunningham et al. 2000), bladder injury (Saban et al. 2007) and cerebral infarction (Junge et al. 2003). These reports strongly support the importance of PAR_1 in orchestrating injury responses. Despite this, PAR_1-

deficiency in mice does not appear to adversely affect normal physiology in adult mice, and no specific phenotypic features have been thus far ascribed to adult $PAR_1^{-/-}$ mice in the literature, although importantly $PAR_1^{-/-}$ mice do have a defect in - endothelial vascular development which is associated with a 50% intraembryonic lethality (Griffin et al. 2001). In addition, gene expression profiling studies have demonstrated that less than 0.5% of genes are differentially regulated in uninjured lungs of $PAR_1^{-/-}$ and WT mice (personal communication and unpublished data, Dr Chris J Scotton, Centre for Respiratory Research). Nevertheless, given that the results of this thesis demonstrated a reduction in lung levels of CCL2, CTGF, and TGF-β_1 at baseline and/or in response to bleomycin in $PAR_1^{-/-}$ mice, and that these mediators have all been associated with deposition of extracellular matrix proteins, it was conceivable that their chronic deficiency might be associated with compromised extracellular matrix protein deposition and the development of emphysema over time. In a similar vein, Bonniaud *et al* previously showed that mice deficient in SMAD3, a pivotal signalling intermediate of TGF-β, developed spontaneous emphysema (Bonniaud et al. 2004).

On visual inspection of histological sections of WT and $PAR_1^{-/-}$ mice, there was no difference between genotypes. A degree of airspace enlargement and scattered peribronchiolar inflammatory infiltrates were present in both genotypes, and most likely reflect usual age-related changes. Hence this data suggests that targeting this receptor would not adversely influence matrix turnover in areas of uninjured lung. This data could be further validated by assessing whether $PAR_1^{-/-}$ mice are more prone than WT mice to the development of emphysema following exposure to tobacco smoke.

4.9 Summary and Clinical Implications

The data presented in this thesis demonstrate that $PAR_1^{-/-}$ mice exhibit attenuated inflammatory and fibrotic responses to bleomycin-induced lung injury. This protection is associated with reduced lung levels of CCL2, CTGF, and TGF-β_1. The effect is apparrently independent of the generation of fibrin, and these results support the notion that PAR_1-dependent generation of these mediators may contribute to inflammation and fibrosis following lung injury. Moreover epithelial cells express

PAR_1 in the injured murine lung, and activation of PAR_1 *in vitro* on both murine and human lung epithelial cells induces the expression of CCL2 and CTGF, but not TGF-β_1. These results therefore support the stated hypothesis of this work, namely that PAR_1 activation promotes lung injury and fibrosis via the release of a number of specific secondary mediators; with the pulmonary epithelium representing an important cellular source of these mediators.

Following epithelial injury, hyperplastic type II alveolar epithelial cells have the potential to differentiate into type I cells, and then spread. Such re-epithelialisation, is part of a normal healing response. Alternatively, hyperplastic type II alveolar epithelial cells can also persist in an activated phenotype and serve as a source of pathological pro-inflammatory and pro-fibrotic mediators. It is tempting to speculate that PAR_1 activation on such cells by locally generated coagulation proteinases might relatively favour the persistence of an activated phenotype with the potential for exaggerated production of secondary mediators, and abnormal interactions with underlying mesenchymal cells. This would relatively favour the development of fibrosis rather than re-epithelialisation and healing. This hypothetical sequence of events is illustrated in **figure 4.1**.

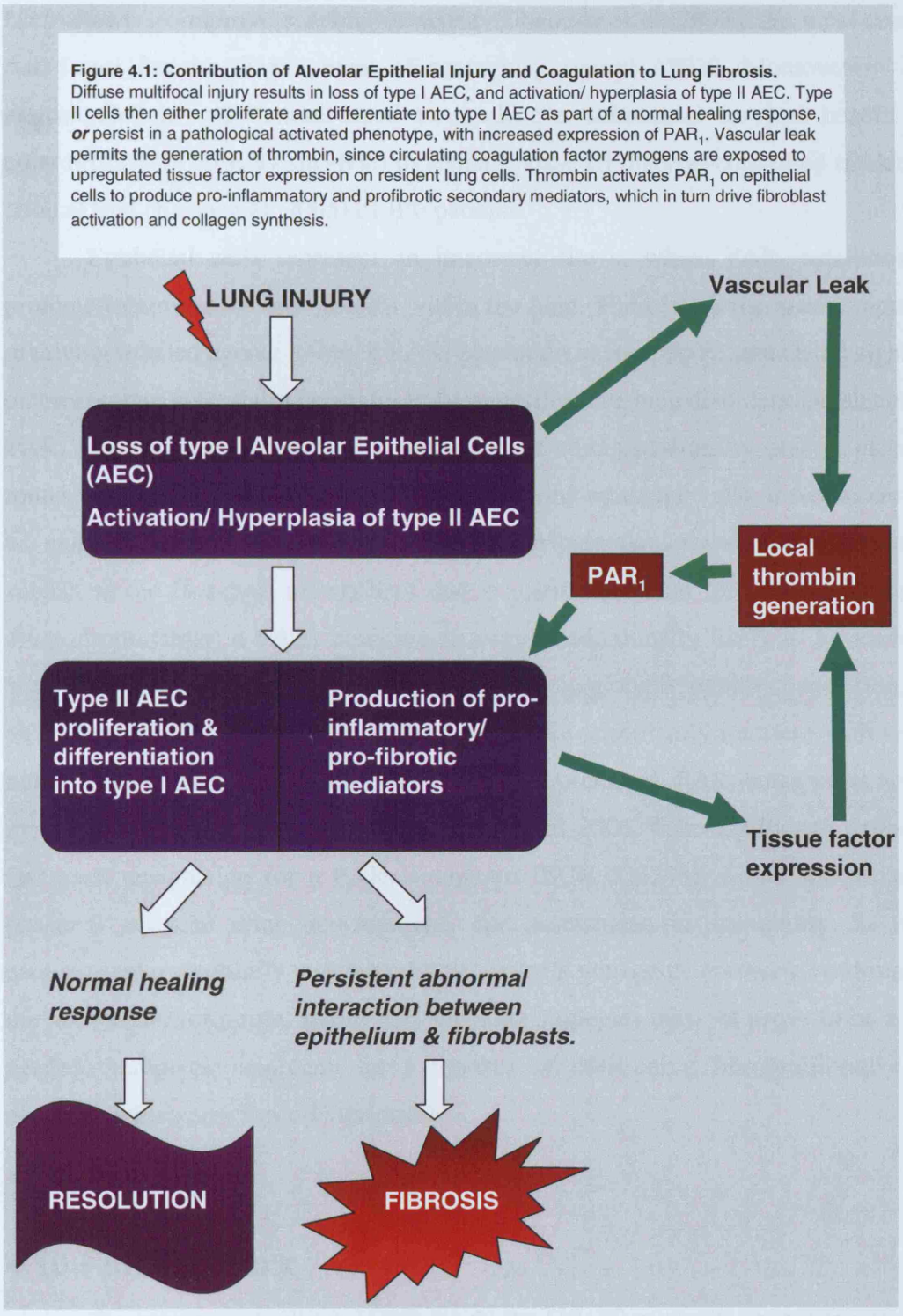


Figure 4.1: Contribution of Alveolar Epithelial Injury and Coagulation to Lung Fibrosis. Diffuse multifocal injury results in loss of type I AEC, and activation/ hyperplasia of type II AEC. Type II cells then either proliferate and differentiate into type I AEC as part of a normal healing response, ***or*** persist in a pathological activated phenotype, with increased expression of PAR_1. Vascular leak permits the generation of thrombin, since circulating coagulation factor zymogens are exposed to upregulated tissue factor expression on resident lung cells. Thrombin activates PAR_1 on epithelial cells to produce pro-inflammatory and profibrotic secondary mediators, which in turn drive fibroblast activation and collagen synthesis.

This work has important implications for human fibroproliferative lung disorders. Evidence for the importance of coagulation in the pathogenesis of fibroproliferative lung disorders is growing. The anticoagulant activated protein C has

been shown to improve survival in sepsis (Dhainaut et al. 2003), the most common risk factor for the development of acute lung injury/ ARDS. Moreover in 2005, anticoagulation was demonstrated to produce a substantial survival benefit over control (63% vs 35% 3y survival) in a small phase III placebo-controlled randomised clinical trial (Kubo et al. 2005) of IPF patients.

Epithelial cells represent an important site at which PAR_1 activation can promote inflammation and fibrosis within the lung. Therefore a therapeutic approach involving inhaled agents to block PAR_1 activation or its specific associated signalling pathways may potentially be useful in fibroproliferative lung disorders. Inhalation of a PAR_1 blocking agent could be beneficial over administration by oral or parenteral routes by delivering the drug more directly to lung epithelial cells. It would certainly be more convenient than a drug given by the parenteral route, and would not be subject to the first-pass metabolism that is often a problem for orally administered drugs. Importantly, a PAR_1 blocking strategy is additionally likely to be associated with less bleeding complications than a strategy to globally inhibit coagulation, such as by warfarin, since PAR_1 blockade would more specifically interfere with cellular rather than procoagulant effects of coagulation proteinases. PAR_1 antagonists are now increasingly credible therapeutic agents. In April 2006, Schering-Plough announced fast track designation for a PAR_1 antagonist (SCH 530348), which was already in phase II clinical drug development, for assessment in its ability to reduce cardiovascular morbidity and mortality in patients with acute coronary syndromes. In the not too distant future, such PAR_1 blocking strategies may yet prove to be a much needed therapeutic approach for a number of devastating fibroproliferative lung disorders which are currently untreatable.

4.10 Further work

To extend the findings of this work, the following specific experiments would be useful. Some would require the generation of transgenic mice and were therefore beyond the scope of this thesis.

1. Assessment of the difference in inflammation and fibrosis between $PAR_1^{-/-}$ and WT mice at 21 days following bleomycin. It is possible that attenuated fibrosis

at 14 days results, at least in part, from an attenuated early inflammatory response. A similar, or greater, attenuation at the later 21 day time-point would further support that PAR_1 deficiency is associated with attenuation of fibrosis, since this time-point is temporally more distant from the early bleomycin-induced inflammation.

2. Evaulation of whether a PAR_1 antagonist confers protection from bleomycin-induced inflammation and/or fibrosis in WT mice. Administration of the antagonist during different stages following bleomycin may shed light on the importance of PAR_1 activation specifically to early inflammatory and to later fibrotic events. Unfortunately at the time of this thesis, lack of compound availability prevented this from being possible

3. Characterization of mechanisms by which PAR_1 expression is regulated in lung epithelial cells. Exposure of lung epithelial cells *in vitro* to mediators such as IL-1b, TNF-α and TGF-β_1 (which have been shown to influence PAR_1 expression in other cell-types), and subsequent assessment of PAR_1 mRNA levels may reveal whether these factors influence PAR_1 expression.

4. Evaluation of whether overexpression of PAR_1 under the influence of an epithelial specific promoter (e.g. SPC) in WT mice is associated with increased bleomycin-induced injury and fibrosis. A similar approach could be undertaken to determine the effect of epithelial re-expression of PAR_1 in $PAR_1^{-/-}$ mice.

5. Evaluation of whether re-expression of CCL2 or CTGF which are deficient in $PAR_1^{-/-}$ mice, restores susceptibility of $PAR_1^{-/-}$ mice to bleomycin-induced fibrosis. These genes could be specifically re-expressed on epithelial cells using an approach such as that described above, or in a less specific manner within the lung. This approach would further support the *in vivo* relevance of CCL2 and CTGF as important PAR_1 secondary mediators in this model.

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6. APPENDIX

A.1: Protection from bleomycin-induced inflammation and fibrosis in $PAR_1^{-/-}$ mice (KO): pilot data from Dr David Howell

A.1.1. BALF inflammatory cells 7 days post saline/ bleomycin:

	WT saline (n=10)	WT bleo (n=12)	KO saline (n=8)	KO bleo (n=13)
Mean cell no. (x 10^4 ± SEM):	13.2 ± 2.5	77.4 ± 116.6 p<0.05 vs WT saline	10.2 ± 15.1	34.2 ± 77.5 p=ns vs KO saline p<0.05 vs WT bleo

A.1.2. BALF protein 7 days post saline/ bleomycin:

	WT saline (n=10)	WT bleo (n=11)	KO saline (n=8)	KO bleo (n=13)
Mean BALF protein (mg/ml ± SEM):	52.3 ± 17.4	818.9 ± 163.1 p<0.05 vs WT saline	14.6 ± 9.5	384.3 ± 96.7 p<0.05 vs KO saline p<0.05 vs WT bleo

A.1.3. BALF collagen (as measured by HPLC) 14 days post saline/ bleomycin:

	WT saline (n=15)	WT bleo (n=14)	KO saline (n=14)	KO bleo (n=13)
Mean collagen (mg/lung ± SEM):	1.42 ± 0.05	2.38 ± 0.07 p<0.01 vs WT saline	1.47 ± 0.09	1.82 ± 0.12 p=ns vs KO saline p<0.05 vs WT bleo

A2: Confirmatory experiment showing attenuation in lung weight increase following bleomycin in $PAR_1^{-/-}$ mice (non-lavaged lungs)

Lung weight in non-lavaged lungs 6 days post saline/ bleomycin:

	WT saline (n=9)	**WT bleo (n=11)**	**KO saline (n=9)**	**KO bleo (n=9)**
Mean lung weight (mg ± SEM)	157.3 ± 7.7	271.5 ± 16.1 p<0.01 vs WT saline	143.7 ± 4.7	218.6 ± 17.8 p<0.01 vs KO saline p<0.01 vs WT bleo

A.3: Confirmatory experiments showing thrombin and TFLLR-NH_2 increase release of CCL2 protein from A549 cells

A.3.1. Effect of thrombin (10nM) on CCL2 protein release into culture supernatants of A549 cells:

	Control medium (n=4)	**thrombin (n=4)**
Supernatant CCL2 after 48h (pM ± SEM)	82.2 ± 2.8	165.4 ± 1.5 p<0.01 vs control

A.3.2. Effect of TFLLR-NH_2 (200μM) and FTLLR-NH_2 (200μM) on CCL2 protein release into culture supernatants of A549 cells:

	Control medium (n=4)	**TFLLR (n=4)**	**FTLLR (n=4)**
Supernatant CCL2 after 6h (pM ± SEM)	20.9 ± 0.3	77.4 ± 0.9 p<0.01 vs control p<0.01 vs FTLLR	19.8 ± 0.6

A.3.3. Effect of thrombin (10nM), Factor Xa (25nM), TFLLR-NH_2 (200μM) and FTLLR-NH_2 (200μM) on CCL2 protein release into culture supernatants of A549 cells:

	Control medium (n=6)	**thrombin (n=6)**	**factor Xa (n=6)**	**TFLLR (n=6)**	**FTLLR (n=6)**
Supernatant CCL2 after 6h (pM ± SEM)	23.5 ± 1.2	62.8 ± 2.0 p<0.01 vs control	39.6 ± 2.0 p<0.01 vs control	84.5 ± 0.9 p<0.01 vs control p<0.01 vs FTLLR	28.2 ± 0.6

A.4: Confirmatory experiments showing PAR_1 antagonist inhibits thrombin-induced increases in CCL2 mRNA levels in A549 and BEAS-2B cells

A.4.1. Effect of RWJ-58259 on thrombin-induced increases in CCL2 mRNA in A549 cells:

[RWJ-58259] (μM)	**0 (n=4)**	**2 (n=4)**	**4 (n=4)**	**6 (n=4)**
% (± SEM) of maximum thrombin-induced CCL2 mRNA levels	100 ± 10.8	8.4 ± 3.2 p<0.01 vs media control / drug vehicle	14.0 ± 1.7 p<0.01 vs media control / drug vehicl	13.3 ± 1.2 p<0.01 vs media control / drug vehicle

A.4.2. Effect of RWJ-58259 on thrombin-induced increases in CCL2 mRNA in BEAS-2B cells:

[RWJ-58259] (μM)	**0 (n=4)**	**2 (n=4)**	**4 (n=4)**	**6 (n=4)**
% (± SEM) of maximum thrombin-induced CCL2 mRNA levels	100 ± 11.6	69.3 ± 5.5	67.8 ± 9.8	39.5 ± 6.7 p<0.01 vs media control / drug vehicle

A.5: Further experiments showing thrombin- or TFLLR-NH_2-induced increases in CCL2 mRNA levels in A549 cells are unaffected by pertussis toxin, but inhibited by the PKC inhibitor Ro-318425

A.5.1. Effect of pertussis toxin on TFLLR-NH_2-induced increases in CCL2 mRNA in A549 cells:

[Pertussis toxin] (ng/ml)	**0 (n=4)**	**100 (n=4)**
% (± SEM) of maximum TFLLR-induced CCL2 mRNA levels	100 ± 18.6	85.5 ± 4.4 p=ns vs media control / drug vehicle

A.5.2. Effect of pertussis toxin on thrombin-induced increases in CCL2 mRNA in A549 cells:

[Pertussis toxin] (ng/ml)	**0 (n=4)**	**100 (n=4)**
% (± SEM) of maximum thrombin-induced CCL2 mRNA levels	100 ± 34.5	73.8 ± 15.9 p=ns vs media control / drug vehicle

A.5.3. Effect of Ro-318425 on TFLLR-NH_2-induced increases in CCL2 mRNA in A549 cells:

[Ro-318425] (μM)	**0 (n=3)**	**1 (n=3)**
% (± SEM) of maximum TFLLR-induced CCL2 mRNA levels	100 ± 10.4	21.4 ± 3.9 p<0.01 vs media control / drug vehicle

A.6: Confirmatory experiments showing thrombin and TFLLR-NH_2 increase release of CCL2 protein from primary human alveolar epithelial cells

A.6.1. Effect of thrombin (10nM), TFLLR-NH_2 (200µM) and FTLLR-NH_2 (200µM) on CCL2 protein release into culture supernatants of primary alveolar epithelial cells (***donor 2***):

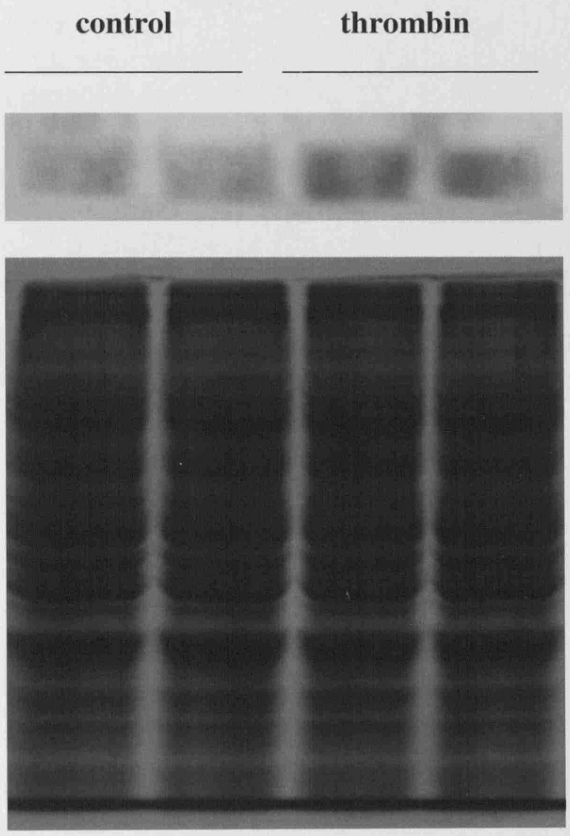
	Control medium (n=6)	**thrombin (n=6)**	**TFLLR (n=6)**	**FTLLR (n=6)**
Supernatant CCL2 after 6h (pM ± SEM)	88.1 ± 9.2	121.6 ± 3.5 p<0.05 vs control	111.8 ± 7.8 p<0.05 vs control	99.6 ± 5.8 p=ns vs control

A.6.2. Effect of thrombin (10nM), factor Xa (25nM), and TFLLR-NH_2 (200µM) on CCL2 protein release into culture supernatants of primary alveolar epithelial cells (***donor 3***):

	Control medium (n=6)	**thrombin (n=6)**	**factor Xa (n=6)**	**TFLLR (n=6)**
Supernatant CCL2 after 6h (pM ± SEM)	1063.6 ± 7.2	1138.9 ± 21.6 p<0.05 vs control	1199.9 ± 36.9 p<0.05 vs control	1242.1 ± 5.6 p<0.05 vs control

A.7: Additional experiment showing thrombin increases CTGF protein in epithelial cells (by western blot)

Figure shows immunreactive 38kDa band obtained after western blotting of lysates of A549 epithelial cells with anti-CTGF polyclonal antibody. Cells were exposed to 25nM thrombin for 4 hours. Coomassie staining of gel ran in parallel confirms approximate equal protein loading between samples.



A.8: Confirmatory experiment showing TFLLR-NH_2 increases CTGF mRNA levels in A549 cells

	Time-point			
	1.5h	**3h**	**6h**	**12h**
Normalised CTGF mRNA levels relative to time-point matched media control (± SEM)	4.37 ± 0.65	4.10 ± 0.20	1.12 ± 0.09	0.86 ± 0.12
Normalised CTGF mRNA of media control (± SEM)	1.01 ± 0.18	1.00 ± 0.10	1.00 ± 0.06	1.00 ± 0.08
	p<0.01	p<0.01	p=ns	p=ns

A.9: Confirmatory experiments showing PAR_1 antagonist inhibits thrombin-induced increases in CTGF mRNA levels in A549 and BEAS-2B cells

A.9.1. Effect of RWJ-58259 on thrombin-induced increases in CTGF mRNA levels in A549 cells:

[RWJ-58259] (μM)	**0 (n=4)**	**2 (n=4)**	**4 (n=4)**	**6 (n=4)**
% (± SEM) of maximum thrombin-induced CTGF mRNA levels	100 ± 10.9	59.9 ± 12.2 p<0.05 vs media control / drug vehicle	51.1 ± 9.9 p<0.05 vs media control / drug vehicle	27.2 ± 7.9 p<0.01 vs media control / drug vehicle

A.9.2. Effect of RWJ-58259 on thrombin-induced increases in CTGF mRNA levels in BEAS-2B cells:

[RWJ-58259] (μM)	**0 (n=4)**	**2 (n=4)**	**4 (n=4)**	**6 (n=4)**
% (± SEM) of maximum thrombin-induced CTGF mRNA levels	100 ± 0.83	81.0 ± 7.0 p<0.05 vs media control / drug vehicle	88.4 ± 2.9 p<0.01 vs media control / drug vehicle	71.5 ± 4.4 p<0.01 vs media control / drug vehicle

A.10: Confirmatory experiments showing thrombin- or TFLLR-NH_2-induced increases in CTGF mRNA levels in A549 cells are unaffected by pertussis toxin, but inhibited by the PKC inhibitor Ro-318425

A.10.1. Effect of pertussis toxin on TFLLR-NH_2-induced increases in CTGF mRNA levels in A549 cells:

[Pertussis toxin] (ng/ml)	**0 (n=4)**	**100 (n=4)**
% (± SEM) of maximum TFLLR-induced CTGF mRNA levels	100 ± 18.2	67.2 ± 11.3 p=ns vs media control / drug vehicle

A.10.2. Effect of pertussis toxin on thrombin-induced increases in CTGF mRNA levels in A549 cells:

[Pertussis toxin] (ng/ml)	**0 (n=4)**	**100 (n=4)**
% (± SEM) of maximum thrombin-induced CTGF mRNA levels	100 ± 20.4	92.6 ± 9.4 p=ns vs media control / drug vehicle

A.10.3. Effect of Ro-318425 on TFLLR-NH_2-induced increases in CTGF mRNA levels in A549 cells:

[Ro-318425] (μM)	**0 (n=3)**	**1 (n=3)**
% (± SEM) of maximum TFLLR-induced CTGF mRNA levels	100 ± 8.4	66.0 ± 0.4 p<0.05 vs media control / drug vehicle

A.11: Three separate experiments confirm that thrombin increases the release of total TGF-β from lung epithelial cells

A.11.1. Thrombin dose response on total supernatant TGF-β levels in mink lung epithelial cell cultures (18h):

	[Thrombin] (nM)						
	control (n=4)	**0.1 (n=4)**	**1 (n=4)**	**10 (n=4)**	**25 (n=4)**	**50 (n=4)**	**100 (n=4)**
Supernatant total TGFβ (ng/ml ± SEM)	1.88 ± 0.11	1.81 ± 0.08	1.90 ± 0.06	2.47 ± 0.10	2.58 ± 0.08	3.26 ± 0.08	4.11 ± 0.29
				p<0.01 vs media control	p<0.01	p<0.01	p<0.01

A.11.2. Thrombin dose response on total supernatant TGF-β of A549 cell cultures (18h):

(Note that pre-incubation of samples with 1D11 pan-TGF-β antibody (100μg/ml) reduces measurable TGF-β to zero, confirming specificity of assay for TGF-β).

	[Thrombin] (nM)							
	control (n=4)	**0.1 (n=4)**	**1 (n=4)**	**10 (n=4)**	**25 (n=4)**	**50 (n=4)**	**100 (n=4)**	**100 + 1D11**
Supernatant total TGFβ (ng/ml ± SEM)	1.28 ± 0.07	1.87 ± 0.08	1.96 ± 0.18	2.17 ± 0.09	2.10 ± 0.12	2.23 ± 0.08	2.33 ± 0.13	0.00
		p<0.01 vs media control	p<0.05	p<0.01	p<0.01	p<0.01	p<0.01	

A.11.3. Thrombin time-course on total supernatant TGF-β of A549 cell cultures (10nM thrombin):

		Time-point						
		3h (n=4)	**6h (n=4)**	**9h (n=4)**	**12h (n=4)**	**18h (n=4)**	**24h (n=4)**	**48h (n=4)**
Supernatant total TGFβ (ng/ml ± SEM)	**Control media**	0.05 ± 0.00	0.06 ± 0.01	0.11 ± 0.00	0.13 ± 0.01	0.36 ± 0.05	0.49 ± 0.04	1.84 ± 0.13
	Thrombin	0.07 ± 0.02	0.16 ± 0.01	0.25 ± 0.11	0.30 ± 0.05	0.45 ± 0.02	0.72 ± 0.06	2.06 ± 0.03
		p=ns vs time-point matched control	p<0.01 vs time-point matched control	p=ns vs time-point matched control	p<0.05 vs time-point matched control	p=ns vs time-point matched control	p<0.05 vs time-point matched control	p=ns vs time-point matched control

A.12: Five separate experiments confirming that factor Xa increases the release of total TGF-β from lung epithelial cells

A.12.1. Effect of 25nM Factor Xa on total supernatant TGF-β of A549 cells (18h):

	Control medium (n=6)	**25nM FXa (n=6)**
Supernatant total TGFβ (ng/ml ± SEM)	0.26 ± 0.05	0.68 ± 0.18 p<0.01 vs control

A.12.2. Effect of 25nM Factor Xa on total supernatant TGF-β of A549 cells (18h):

	Control medium (n=6)	**25nM FXa (n=6)**
Supernatant total TGFβ (ng/ml ± SEM)	0.21 ± 0.02	0.50 ± 0.06 p<0.01 vs control

A.12.3. Effect of 25nM Factor Xa on total supernatant TGF-β of A549 cells (18h – measured by ELISA):

	Control medium (n=9)	**25nM FXa (n=9)**
Supernatant total TGFβ (ng/ml ± SEM)	0.11 ± 0.08	0.38 ± 0.01 p<0.01 vs control

A.12.4. Effect of 10nM and 25nM Factor Xa on total supernatant TGF-β of A549 cells:

	Control medium (n=6)	**10nM FXa (n=6)**	**25nM FXa (n=6)**
Supernatant total TGFβ (ng/ml ± SEM)	0.14 ± 0.02	0.29 ± 0.02 p<0.01 vs control	0.47 ± 0.03 p<0.01 vs control

A.12.5. Factor Xa time-course on total supernatant TGF-β of A549 cells (25nM FXa):

		Time-point					
		3h (n=3)	**6h (n=3)**	**12h (n=3)**	**18h (n=3)**	**24h (n=3)**	**48h (n=3)**
Supernatant total TGFβ (ng/ml ± SEM)	**Control media**	0.04 ± 0.00	0.05 ± 0.02	0.07 ± 0.01	0.21 ± 0.01	0.30 ± 0.02	0.18 ± 0.00
	Factor Xa (25nM)	0.14 ± 0.03	0.18 ± 0.01	0.28 ± 0.04	0.77 ± 0.04	1.04 ± 0.07	0.95 ± 0.10
		p<0.05 vs time-point matched control	P<0.01 vs time-point matched control	p<0.01 vs time-point matched control	p<0.01 vs time-point matched control	p<0.01 vs time-point matched control	p<0.01 vs time-point matched control

A.13: Two separate experiments confirm that factor Xa and TFLLR-NH_2, but not thrombin exert a small stimulatory effect on total TGF-β release from primary human lung alveolar epithelial cells

A.13.1. Effect of thrombin, factor Xa, and TFLLR-NH_2 on total supernatant TGF-β of primary alveolar epithelial cells after 18 h (***donor 1***):

	Control medium (n=6)	**10nM thrombin (n=6)**	**25nM Fxa (n=6)**	**200μM TFLLR (n=6)**
Supernatant total TGFβ (ng/ml ± SEM)	2.51 ± 0.14	2.90 ± 0.13	2.89 ± 0.10	2.99 ± 0.11
		p=0.06 vs control	p<0.05 vs control	p<0.01 vs control

A.13.2. Effect of thrombin, factor Xa, and TFLLR-NH_2 on total supernatant TGF-β of primary alveolar epithelial cells after 18 h (***donor 3***):

	Control medium (n=6)	**10nM thrombin (n=6)**	**25nM Fxa (n=6)**	**200μM TFLLR (n=6)**
Supernatant total TGFβ (relative luminescence units ± SEM)	8900.2 ± 379.8	9935.4 ± 344.4	10060 ± 252.6	10831.5 ± 417.9
		p=0.07 vs control	p<0.05 vs control	p<0.01 vs control

A.14: Two further experiments showing that TFLLR-NH_2 and FTLLR-NH_2 *both* increase release of total TGF-β from lung epithelial cells

A.14.1. Effect of TFLLR-NH_2 and FTLLR-NH_2 on total supernatant TGF-β of A549:

	Control medium (n=5)	**TFLLR-NH_2 (n=7)**	**FTLLR-NH_2 (n=7)**
Supernatant total TGFβ (relative luminescence units ± SEM)	0.35 ± 0.05	1.29 ± 0.08 p<0.01 vs control	0.84 ± 0.05 p<0.01 vs control

A.14.2. Effect of TFLLR-NH_2 and FTLLR-NH_2 on total supernatant TGF-β of A549:

	Control medium (n=5)	**TFLLR-NH_2 (n=7)**	**FTLLR-NH_2 (n=7)**
Supernatant total TGFβ (relative luminescence units ± SEM)	0.20 ± 0.04	0.91 ± 0.06 p<0.01 vs control	0.68 ± 0.05 p<0.01 vs control

A.15: Further experiment showing TFLLR-NH_2 has little effect on TGF-β_1 mRNA levels in A549 cells

	Time-point			
	1.5h	**3h**	**6h**	**12h**
Normalised TGF-β_1 mRNA control relative to time-point matched control (± SEM)	1.22 ± 0.09	1.15 ± 0.06	1.13 ± 0.04	1.41 ± 0.12
Normalised TGF-β_1 mRNA of control (± SEM)	1.01 ± 0.08	1.00 ± 0.03	1.00 ± 0.04	1.01 ± 0.08
	p=0.14	p=0.06	p=0.05	p<0.05

A16: Publications, presentations and prizes relating to this work

Publications

Absence of Proteinase-Activated Receptor-1 Signaling affords Protection from Experimentally-Induced Lung Inflammation and Fibrosis. **RH Johns**, DCJ Howell, JA Lasky, B Shan, CJ Scotton, GJ Laurent, RC Chambers. *American Journal of Pathology May 2005, 166(5), 1353-65*

Manuscript under review:
The Hyperplastic Epithelium Represents a Prominent Source of PAR_1-inducible MCP-1/CCL2 in Pulmonary Fibrosis. **RH Johns**, PF Mercer, CJ Scotton, MA Krupiczojc, M Koenigshoff, RJ McAnulty, A Das, O Eikelberg, RC Chambers

Manuscript in preparation:
Protection from Bleomycin-induced Lung Inflammation and Fibrosis in PAR_1-deficient Mice is Asscoiated with Reduced Upregulation of Multiple Cytokine Networks. **RH Johns**, A Naldini, C Ardinghi, F Carraro, RC Chambers

Published abstracts (reverse chronological order)

PAR_1 Activation on Lung Epithelial Cells Promotes CCL2 Expression. **RH Johns**, CJ Scotton, AJ Thorley, GJ Laurent, TD Tetley, RC Chambers. *Am J Resp Crit Care Med ATS supp 2006*

PAR_1 Activation on Airway and Alveolar Epithelial Cells Induces production of CTGF. CJ Scotton, **RH Johns**, GJ Laurent, RC Chambers. *Am J Resp Crit Care Med ATS supp 2006*

PAR_1 Signalling in Lung Epithelial Cells Induces the Expression of CCL2/MCP-1 and CTGF. **RH Johns**, CJ Scotton, GJ Laurent, RC Chambers. *Thorax supp. II, Dec 2005*

Protection from Bleomycin-Induced Lung Injury in the Absence of PAR_1 Signaling. **RH Johns**, DCJ Howell, B Shan, J Lasky, GJ Laurent, RC Chambers. *Am J Resp Crit Care Med ATS supp. 2005*

Transcriptional profiling reveals reduced expression of inflammatory genes in the lungs of PAR_1-deficient mice, seven days after bleomycin-induced lung injury. CJ Scotton, YCG Lee, **RH Johns**, GJ Laurent, RC Chambers. *Am J Resp Crit Care Med ATS supp 2005*

Bleomycin-induced Transcriptional Responses are Globally Blunted in Protease Activated Receptor (PAR)-1 Knockout Mice at 14 Days. YCG Lee, CJ Scotton, **RH Johns**, RC Chambers. *Am J Resp Crit Care Med ATS supp. 2005*

Role of Proteinase Activated Receptor-1 in the Development of Goblet Cell Metaplasia in FMLP-Treated Mice. L Atzori, M Lucatelli, B Bartalesi, B Lunghi, **RH Johns**, R Chambers, G Lungarella. *Am J Resp Crit Care Med ATS supp 2005*

Global Gene Expression Profiling of the Lungs of Proteinase Activated Receptor (PAR)-1 Deficient Mice Following Bleomycin-Induced Lung Injury. YCG Lee, CJ Scotton, **RH Johns**, GJ Laurent, RC Chambers. *Respirology, 10 supp. 2005*

Absence of Proteinase-Activated Receptor-1 Signalling Affords Protection from Experimentally Induced Lung Injury and Fibrosis. **RH Johns**, DCJ Howell, GJ Laurent, RC Chambers. *Thorax supp. II, Dec 2004*

Role of Thrombin-Induced TGF-β Release in Experimental Pulmonary Fibrosis. **RH Johns**, DCJ Howell GJ Laurent RC Chambers. *Am J Resp Crit Care Med ATS supp. 2004*

Reduced levels of Connective Tissue Growth Factor (CTGF) in the Protease Activated Receptor-1- (PAR1)- Knockout Mouse. **RH Johns**, DCJ Howell GJ Laurent RC Chambers. *Am J Resp Crit Care Med ATS supp. 2003*

Presentations (reverse chronological order)

Abstract & Poster Presentation: *PAR_1-deficiency Affords Protection from Experimentally-Induced Lung Injury and Fibrosis.* **RH Johns**, DCJ Howell, GJ Laurent, RC Chambers. (Presented at Medical Research Society meeting, Royal College of Physicians London, February 2005)

Abstract & Oral Presentation: *PAR_1 Signalling in Lung Epithelial Cells Induces the Expression of CCL2/MCP-1 and CTGF.* **RH Johns**, CJ Scotton, GJ Laurent, RC Chambers. (Presnted at British Thoracic Society Symposium: Novel molecular mechanisms of disease, BTS Winter Meeting 2005; published in *Thorax supp. II, Dec 2005*)

Abstract & Oral Presentation: *Absence of Proteinase-Activated Receptor-1 Signalling Affords Protection from Experimentally Induced Lung Injury and Fibrosis.* **RH Johns**, DCJ Howell, GJ Laurent, RC Chambers. (Runner up in BTS Young Investigator Symposium, BTS Winter Meeting 2004; published in *Thorax supp. II, Dec 2004*)

Abstract & Oral Presentation: *Protection from Bleomycin-Induced Lung Injury and Fibrosis in Proteinase-Activated Receptor-1-Deficient ($PAR_1^{-/-}$) Mice is Associated with Reduced Expression of Pro-Inflammatory and Pro-Fibrotic Mediators.* **RH Johns**, DCJ Howell, GJ Laurent, RC Chambers, (Presented at 13th International Colloquium on Lung Fibrosis, Banff, Canada, 2004)

Poster Presentation: *Finding a Cure for Lung Fibrosis?* **RH Johns** (Prize-winner in "Perspectives" Poster Competition, BA Festival of Science, Exeter, UK, September, 2004)

Abstract & Poster Presentation: *Role of Thrombin-Induced TGF-β Release in Experimental Pulmonary Fibrosis.* **RH Johns**, DCJ Howell GJ Laurent RC Chambers, (Presented at ATS 2004. Published in *Am J Resp Crit Care Med ATS supp.* 2004)

Abstract & Poster Presentation: *PAR_1 Activation Promotes Generation of Pro-fibrotic Mediators in Experimental Lung Injury.* **RH Johns**, DCJ Howell GJ Laurent RC Chambers, (Presented at Medical Research Society meeting, Royal College of Physicians, London May, 2004)

Abstract & Oral Presentation: *Protection From Fibrosis in the PAR_1-Knockout Mouse is Associated with Reduced Epithelial Release of Transforming Growth Factor-beta (TGF-β).* **RH Johns**, DCJ Howell GJ Laurent RC Chambers, (Presented at Young Scientist Competition, Sept 2003, British Association of Lung Research Summer Meeting)

Abstract & Poster Presentation: *Reduced Levels of Connective Tissue Growth Factor (CTGF) in the Protease Activated Receptor-1- (PAR_1)- Knockout Mouse.* **RH Johns**, DCJ Howell GJ Laurent RC Chambers, (Presented at ATS 2003. Published in *Am J Resp Crit Care Med ATS supp.* 2003)

Prizes

MRC Clinical Research Training Fellowship. Feb 2003

UCL Graduate School Research Projects Award 2003

British Lung Foundation/ Allen & Hanburys Travel Fellowship. May 2003

British Association of Lung Research Summer Meeting Travel Award. September 2003

Runner-up Award. "Perspectives" Poster Competition. BA Festival of Science, September 2004

Pfizer Academic Travel Award. October 2004

Runner up Award. Young Investigator Competition, British Thoracic Society Winter Meeting, December 2004

UCL Graduate School Poster Competition Runner-up prize 2005

Lancet Travel award 2005